ANTI-VASCULOGENIC EFFECT OF MYCOPHENOLIC ACID

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DEDICATION

To my husband, for his unconditional love and support.

To my patients, who inspire me to work hard, to keep asking questions and to never stop looking for answers.

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ANTI-VASCULOGENIC EFFECT OF MYCOPHENOLIC ACID

Patients with chronic inflammatory rheumatic disease (CIRD) manifest prominent systemic and local vascular damage. Prolonged inflammation results in widespread endothelial dysfunction with subsequent development of atherosclerosis and premature cardiovascular disease that is becoming prevalent in patients with rheumatic disease. Although inflammation occurs primarily at the endothelium-leukocyte interface, very little is known about the intrinsic vasculoreparative properties of the damaged endothelium. Circulating and tissue resident vascular endothelial progenitor cells (EPC) are believed to participate in repairing the damaged endothelium and maintaining the integrity of the vascular lining. Endothelial colony forming cells (ECFC) are rare, viable, circulating cells that display properties consistent with those of a progenitor cell have been identified in humans. Alterations in the function of ECFC results in impaired vessel repair mechanisms. In this work, we sought to determine the effects of a commonly used immunosuppressive drug, mycophenolic acid (MPA), in treatment of CIRD. MPA inhibits ECFC proliferation in a dose dependent fashion, blunts cell migration, and reduces ECFC vasculogenic function. Consequently, MPA could contribute to endothelial damage in many rheumatic diseases associated with vascular pathology.

The ATP-binding cassette transporter subfamily G member 2 (*Abcg2*), a cell surface marker for stem cell isolation in multiple lineages. Yoder/Basile lab showed that *Abcg2* is highly expressed in tissue vascular EPC using knock-in mice for a tamoxifen-inducible *Abcg2* promoter driven Cre recombinase with *ROSATdTomato* transgenic reporter mice. Preliminary work shows that murine blood vessels contain *Abcg2*-expressing endothelial cells that contribute to heart and kidney vessel growth during development, and

maintenance of kidney vessel homeostasis in adults in vivo. Systemic lupus erythematosus (SLE) is a type of CIRD that is characterized by microvascular inflammation and damage, endothelial dysfunction and increased cardiovascular risk. Future studies are directed towards identifying how lupus-associated factors and mycophenolic acid influence *Abcg2*-expressing endothelial cell properties using a well-described pristane-induced lupus mouse model.

Kathleen M. O'Neil, M.D., Chair

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2D	Two dimendional
3D	Three dimensional
µM	Micromolar
ABC	ATP-binding casette
Abcg2	ATP-binding cassette transporter 2
ABCG2TT	Abcg2CreERT; ROSATdTomato
Ac-LDL	Acetylated low density lipoprotein
AcMPAG	Mycophenolic acid-acyl-glucuronide
ACR	American College of Rheumatology
BCA	Bicinchoninic acid
BrdU	5'-bromo-2'-deoxyuridine
CAC	Circulating angiogenic cells
cDNA	Complementary DNA
CFSE	Carbocyfluorescein succinimidyl ester
CFU-Hill	Colony forming unit-Hill
CIRD	Chronic inflammatory rheumatic disease
cSLE	Childhood-onset systemic lupus erythematosus
CV	Cardiovascular
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole stain
DiI-Ac-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
DMSO	Dimethylsulfoxide
EC	Endothelial cells
ECFC	Endothelial colony forming cells
EGM-2	Endothelial growth medium-2
EPC	Endothelial progenitor cells
ERT2	Estrogen Receptor Type 2
ESRD	End stage renal disease
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GMP	Guanine monophosphate
GTP	Guanine triphosphate
HPP	High proliferative potential
IFN	Interferon
IL	Interleukin
IMP	Inosine-5'-monophosphate
IMPDH	Inosine monophosphate dehydrogenase
ISN/RPS	International Society of Nephrology/Renal Pathology Society
JDM	Juvenile dermatomyositis
LN	Lupus nephritis
LPP	Low proliferative potential
MFI	Mean fluorescence intensity
MMF	Mycophenolate mofetil
MMS	Mycophenolate sodium
MNC	Mononuclear cell
MPA	Mycophenolic acid
MPAG	Mycophenolic acid glucuronide
MRP-2	Multi-drug resistance protein 2
n.s.	Not significant

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Ph	Phosphorylated
PI	Propidium iodide
PSV	Primary systemic vasculitis
qRT-PCR	real time polymerase chain reaction
RNA	Ribonucleic acid
SLE	Systemic lupus erythematosus
SA-β-GAL	Senescence-associated-beat-galactosidase
SSc	Systemic sclerosis
$t_{1/2}$	Half-life
TMPD	2,6,10,14 tetramethylpentadecane
TNF-α	Tumor necrosis factor-alpha
Tot	Total
UEA-1	Ulex Europaeus Agglutinin Type I
UGT	UDP glucuronosyltransferases
VEGF	Vascular endothelial growth factor
VESC	Vascular endothelial stem cells
XMP	Xanthine monophosphate

CHAPTER I

1.1 INTRODUCTION

Chronic inflammatory rheumatic diseases (CIRD) are a group of heterogeneous but related disorders, some of which include systemic lupus erythematosus, juvenile dermatomyositis, systemic sclerosis and systemic vasculitis. Despite having varied clinical manifestations, they all share a common pathologic feature of inflammation mediated by leukocytes at the interface with the lining of blood vessels. Vascular lesions can be the result of vasculitis (transmural inflammation of the blood vessel wall), or vasculopathy, (non-transmural injury to the vessels that is not the result of primary vessel wall inflammation), like thromboembolic disease, coagulative disorder or other forms of vessel injury. Any vessel type can be involved and thus vasculopathy can result in varied phenotypic expression, anywhere from mild cutaneous lesions (rash) to systemic features that compromise the integrity and function of any organ.

1.2 VASCULAR INVOLVEMENT IN CIRD

1.2.1 SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

SLE is a prototypic systemic autoimmune condition mediated by immune-complex induced inflammation. Vascular lesions are among the most characteristic features of SLE and have a prevalence that ranges between 10-40% of SLE patients [1-3]. They may precede the development of full-blown disease and play a key role in the choice of treatment strategy and prognosis. Vascular injury in lupus can cause lupus vasculopathy (vascular wall necrosis and a thrombus in the lumen of an affected artery), lupus vasculitis with inflammation, necrotizing lesions, thrombotic microangiopathy, and atherosclerosis [4, 5]. Cutaneous microangiopathy, classified as small-vessel involvement, are the most common representing 90% [2, 6]; however, widespread,

necrotizing visceral medium-and large-vessel involvement that affects the heart, lungs brain and kidney, may also occur.

1.2.2 JUVENILE DERMATOMYOSITIS (JDM)

JDM is an autoimmune condition of childhood that is characterized by small vessel vasculopathy affecting the skin and musculature but can involve the heart, lungs and gastrointestinal tract as well. Although classified as an inflammatory myopathy, JDM displays prominent vascular and perivascular inflammation that contributes directly to the clinical features and outcome of the disease [7-9]. Biopsy of involved areas reveals extensive reduction in capillary density, endothelial antibody deposition and complement activation that leads to endothelial dysfunction, endothelial release of pro-inflammatory cytokines and upregulation of adhesion molecules [7, 10-13].

1.2.3 SYSTEMIC SCLEROSIS (SSc)

SSc is a connective tissue disease characterized by inflammation, vasculopathy and progressive fibrosis of the skin, lungs and other tissues. SSc is considered a fibrosing disease, but vascular involvement plays a major role in pathogenesis and organ dysfunction. Autopsy studies showed that SSc patients have widespread intimal proliferation of small and/or large arteries in the pulmonary, coronary, and renal circulation [14].The initial vascular injury from autoimmune or environmental factors cause endothelial cell dysregulation vacuolization of endothelial cell cytoplasm, and loss of membrane-bound storage vesicles [15-17]. Vasculopathy then ensues resulting from abnormal vasoreactivity, tissue hypoxia, dysregulation of coagulation, fibrinolytic processes, and/or direct structural damage [18-20]. Impaired compensatory vasculogenesis and angiogenesis plays an important role in the fibroproliferative vasculopathy observed in SSc [16, 19, 21].

1.2.4 PRIMARY SYSTEMIC VASCULITIS (PSV)

PSV is a group of multisystem disorders in which the blood vessels are the major disease target with no other underlying disease and thus, endothelial dysfunction is inherent in these diseases. Disease classification is based on the size of affected vessels; the clinical presentation depends on the site, type and size of vessels involved [22, 23]. Some of the acute primary vasculitis disorders are common pediatric diseases like Henoch-Schonlein purpura and Kawasaki disease, while others are rare like ANCA-associated vasculitis and Takayasu disease [24]. The pathogenesis underlying these inflammatory conditions include cell-mediated inflammation, immune complex-mediated inflammation and autoantibody driven but the central histological feature involves endothelial activation leading to massive inflammatory cell infiltration along the vessel wall, necrosis, thrombosis, aneurysm or vascular narrowing [25, 26].

1.3 CARDIOVASCULAR DISEASE IN CIRD

CIRD has been linked to cardiovascular disorders, with a markedly increased risk of cardiovascular disease (CVD) compared to the general population. Patients with SLE, PSV, JDM and SSc all have high rates of early onset atherosclerosis, premature cardiovascular events and coronary disease [27-32]. The link between rheumatoid arthritis and cardiovascular morbidity has been unequivocally established in historical cohorts [33-36]. As much as 50% of premature deaths in this population is mainly due to increased cardiovascular disease [33, 34, 37]. The reasons for the high CVD prevalence are multifactorial. Patients with CIRD carry more traditional unfavorable cardiovascular risk factors and have disease-related risks through several mechanisms including autoantibodies, autoantigen expressions, autoreactive lymphocytes, epigenetic mechanisms, and inflammatory components driving the formation, progression and rupture of atherosclerotic plaques, and activation of the coagulation system [38-42].

The endothelium has received much attention because it is believed that endothelial dysfunction is one of the earliest steps involved in the process of atherogenesis. The endothelium produces nitric oxide, an endothelium-derived relaxing factor that promotes vasodilation and reduces vascular resistance in response to physiological changes, and also possesses anti-inflammatory and anti-thrombotic properties at the endothelial blood interface [43, 44]. Deficiency in the production and bioavailability of nitric oxide resulting from endothelial damage leads to impairment of endothelial-dependent vasodilation, which has been proven to be an independent risk factor of cardiovascular events [45, 46].

The imbalance between endothelial damage and repair may also lead to increased cardiovascular events [47]. Endothelial progenitor cells (EPC) are believed to participate in repairing the damaged endothelium and maintaining the integrity of the vascular lining. Several studies have reported that inflammation negatively influences the mobilization and differentiation of EPC [48-51]. In many rheumatic diseases, decreased numbers and functionally altered EPCs have been described [52-56].

There remains considerable uncertainty about the effect of immunosuppressive drugs on the risk of CVD in patients with rheumatic disease. Certain immunosuppressive agents have been associated with reduced risk or mortality related to CVD [57-59], while others showed potential cardiovascular toxicities [60]. Various traditional and unconventional cardiovascular risk factors are potentiated by the adverse effects of immunosuppressive drugs.

1.4 IMMUNOSUPPRESSIVE THERAPY

The treatment of inflammatory rheumatic diseases is a major challenge for rheumatologists primarily because the etiology of the majority of rheumatic disease is incompletely understood, involving complex interactions among genetics, environment and host factors, and the heterogeneity of disease phenotypes. We know for a fact that aberrations in immune function are involved in disease pathogenesis and present at all stages of disease. Hence, current therapeutic approaches are largely to restrict immunologic function thereby reducing inflammatory damage.

Systemic immunosuppressive agents used in CIRD include, but are not limited to, glucocorticoids, cyclophosphamide, cyclosporine, tacrolimus, methotrexate, and mycophenolate mofetil [61, 62]. These agents are effective in inducing and maintaining remission of many autoimmune/rheumatic disease. The five-year survival of patients with rheumatic disease has improved dramatically thanks to the use of immunosuppressants [63-65]. The initial drugs of choice used in treating disease, like corticosteroids and cyclophosphamide, are largely non-specific. They interfere with major immune pathways and in a variety of cells. However, this also means more unwanted effects are associated with use of these agents. Recently, more target-specific drugs are becoming available which have potential to reduce the toxicity of immunosuppressant drugs. However, not all patients respond to all immunosuppressants, and these medications do have potentially serious side effects.

1.5 MYCOPHENOLIC ACID

Mycophenolic acid (MPA) is the active drug of mycophenolate mofetil (MMF). It was first discovered by Gosio in 1893 as a fermentation product of *Penicillium* culture which was later confirmed by others [66, 67]. MPA was found to have antimicrobial and antifungal

properties but its anti-infective properties are modest [67, 68]. Through the early experiments, it was discovered that MMF has antimitotic properties raising interest that it might be a good agent for immunosuppressive therapy [69]. Currently, it is widely used in transplantation for prophylaxis of organ rejection. Taking advantage of its effects on the immune system, MPA/MMF is frequently prescribed by rheumatologists to and found to be effective in treating systemic inflammatory diseases such as SLE, polymyositis, dermatomyositis and SSc.

1.5.1 PHARMCOKINETICS

MMF is rapidly absorbed and hydrolyzed to the active compound MPA by plasma and tissue esterase after oral administration. It is highly bound to albumin (97 to 99%) in patients with normal renal and liver function. The plasma concentration of MPA rises rapidly and peaks within 1 hour of ingestion, followed by a second peak at 6 to 12 hours due to enterohepatic circulation. The mean apparent half-life ($t_{1/2}$) of MPA in the systemic circulation is approximately 9 to 17 hours. The majority of MPA is metabolized in the liver (and a small proportion by other tissues, including intestine and kidney) through a phase II glucuronidation process mediated by UDP glucuronosyltransferases (UGT). The major metabolite of MPA is the pharmacologically inactive 7-O-glucuronide metabolite (MPAG), which is produced by UGT1A9. Two other metabolites—MPA-acyl-glucuronide (AcMPAG) and MPA-phenyl-glucoside (glucoside-MPA) – are also produced [70, 71]. (Figure I-1). Inactive phenolic glucuronide is excreted in both urine via active tubular secretion and bile via multi-drug resistance protein 2 (MRP-2). Biliary-excreted MPAG can be deconjugated back to MPA during enterohepatic recirculation which contributes to 40% of area under the plasma concentration-time curve [70]. MPAG is present at a much higher concentration than MPA but it does not have any immunosuppressive activity. AcMPAG was known as pharmacologically inactive however, some investigators

have demonstrated that it has an inhibitory effect on lymphocyte proliferation in vitro and could possibly be associated with toxic effects [72]. The therapeutic concentrations of MPA after oral administration vary between 1 to 10 µM (0.32 to 3.2 mg/L).

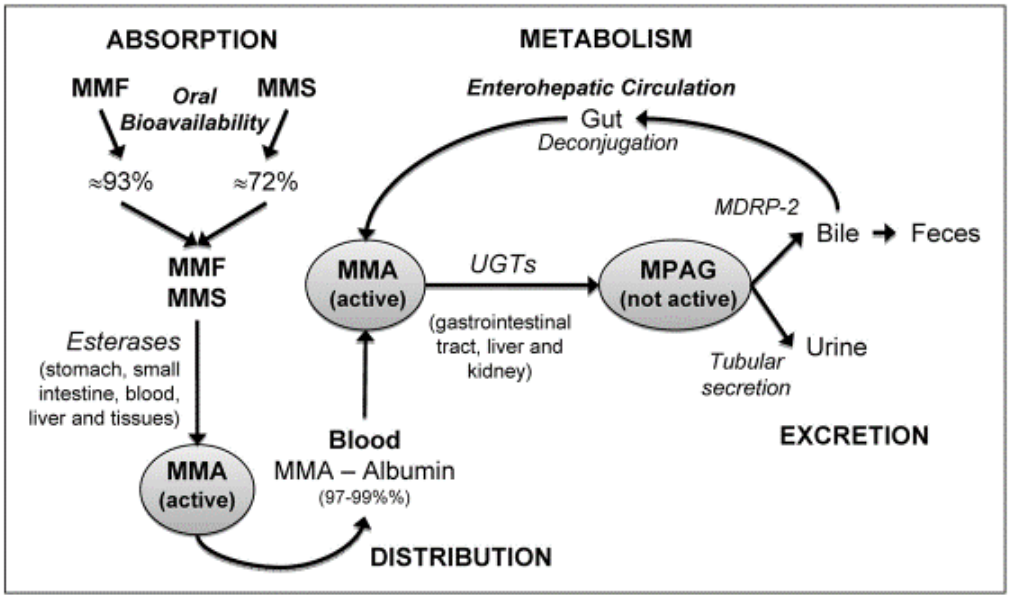


Figure I-1. Mycophenolic Acid Pathway.

Simplified pathway of how MMF and MMS prodrug are absorbed, distributed, metabolized and excreted in the system.

MMF = mycophenolate mofetil, MMS = mycophenolate sodium, MMA = mycophenolic acid, UGT = UDP glucuronosyltransferase. Figure adapted from Perez-Aytes *et. al.* (2007) [73].

1.5.2 MECHANISM OF ACTION

MPA inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH) causing the depletion of the intracellular guanine triphosphate (GTP) supply [74]. IMPDH catalyzes the NAD-dependent formation of the parent compound of purine nucleotides, xanthine monophosphate (XMP) from inosine-5'-monophosphate (IMP). This reaction is

irreversible and a committed step (rate-limiting step) to the synthesis of guanine monophosphate (GMP) [69]. Figure I-2 illustrates how MPA disrupt purine synthesis.

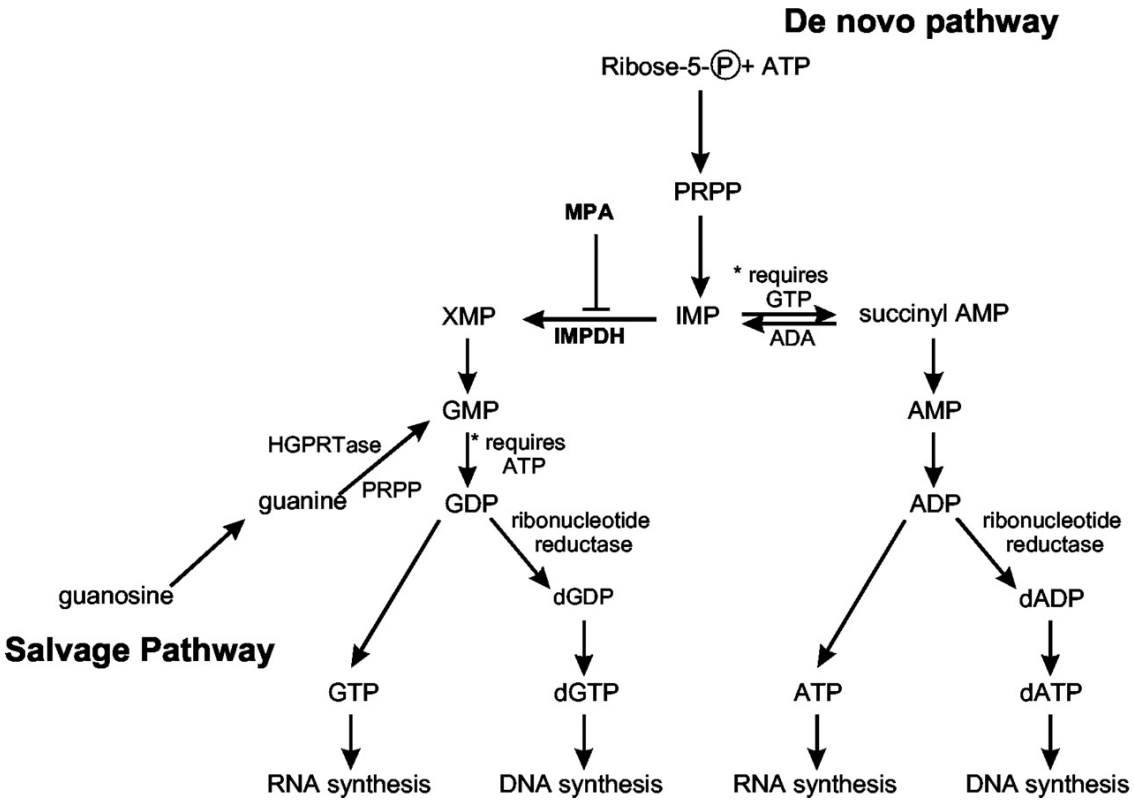


Figure I-2. Pathway of purine biosynthesis.

MPA inhibits IMPDH, which directly causes depletion of the guanine nucleotides GMP, GDP, dGDP, GTP, and dGTP).

MPA = mycophenolic acid, IMPDH = inosine monophosphate dehydrogenase, GMP = guanosine monophosphate, GDP = guanosine diphosphate, GTP = guanosine triphosphate, dGDP = deoxyguanosine diphosphate, dGTP = deoxyguanosine triphosphate, HGPRT = hypoxanthine guanine phosphoribosyltransferase, PRPP = phosphoribosylpyrophosphate. Figure adapted from Hermann *et. al.* (2004) [75].

Most cell types have the capacity to synthesize guanosine nucleotides by the IMPDH and salvage pathways, but lymphocytes are dependent upon de novo synthesis [69]. It is therefore believed that MPA selectively inhibits the proliferation of human T and B

lymphocytes. However, MPA affects many other cell types and does so by non-IMPDH dependent pathways since they are not reversible by guanosine administration [76-79].

1.5.3 EFFECTS OF MPA ON ENDOTHELIAL CELLS

MPA reduces cytokine-induced production of nitric oxide, and cytokine-mediated expression of VCAM-1, ICAM-1, E-selectin, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-6 [79-81]. It has been shown to inhibit endothelial migration, proliferation and angiogenesis *in vitro* [82-84]. In addition, MPA reduces endothelin-1 expression and increases prostacyclin release [82].

1.6 ENDOTHELIUM

The endothelium is a single layer of cells that line the entire vascular system from the arteries to the smallest capillaries. Endothelial cells (EC) are found on the luminal surface of blood vessels where they are metabolically active. Endothelium not only functions as a physical barrier to agents, but it also regulates vascular tone and remodeling, transports various molecules, synthesizes proteins and secretes vasoactive agents and cytokines that are critical in maintaining homeostasis [85]. The properties of the endothelium vary among different sites of the vasculature reflecting different functions within the body [86, 87].

1.6.1 NEW VESSEL FORMATION

EC have the capacity to proliferate and migrate to denuded areas either by angiogenesis or vasculogenesis [88-90]. This begins at the embryonic stage during organ development and continues throughout life to remodel structures and repair damaged vessels. In general, cell division is rapid early in life to keep pace with growth and then slows in adulthood.

Blood vessels can form via two processes. In angiogenesis, new capillaries arise from the wall of existing vessels through a series of coordinated events in response to specific signals [91]. Vasculogenesis, on the other hand, is in situ formation and growth of primitive vessels from primitive angioblasts [91, 92]. It was believed that de novo vessel formation is limited to early embryogenesis and does not occur after the vascular system is established. It was not until two decades ago that the concept of postnatal vasculogenesis as a form of neovascularization was introduced. Human CD34+ cells isolated from circulating peripheral blood and bone marrow differentiated into endothelial cells in vitro and also in vivo that later contributed to neovascularization in the host animal [88, 93]. These cells were called endothelial progenitor cells (EPC) and were found to be present in sites of tissue ischemia and injury. The discovery of EPC sparked great interest in terms of vessel and tissue renewal, and remains a popular area of research in understanding disease pathogenesis and regenerative medicine.

1.6.2 IDENTIFICATION OF CIRCULATING ENDOTHELIAL CELLS INVOLVED IN VESSEL REPAIR

EPC are broadly referred to as cells capable of differentiating into endothelial cells that contribute to the formation of new blood vessels. EPC were first described by Asahara et al. based on surface antigen expression, morphology and ability to incorporate into vessels [93]. This vague definition led to multiple cell types being called EPC, resulting in contradictory findings as to their precise role in health and disease. Hematopoietic and myelomonocytic cells that exhibit endothelial cell properties e.g., expression of endothelial cell surface markers and colony forming activity, when exposed under the right condition have been incorrectly labeled as EPC [94-96]. Therefore, stringent classification and definition is the first necessary step to eliminate confusion and to

permit the field to advance. Experts in this discipline addressed this issue recently and proposed standards to confirm an endothelial progenitor phenotype [97]. EPC phenotype and function should be identified using detailed immunophenotyping, potency assays, and clear separation from hematopoietic angiogenic cells which are not endothelial progenitors.

Distinguishing EPC from mature functional endothelial cells and hematopoietic cells based on cell surface marker expression alone is challenging because these categories of cells commonly share many markers. It is imperative to show that cells have the capacity to differentiate into mature functional endothelial cells in vitro and in vivo requiring the assessment of a variety of parameters including morphology, behavior, in situ localization, and both gene and protein expression [94, 98-100].

Endothelial cells grow in monolayers in vitro, become growth inhibited on reaching confluence, and exhibit a “cobblestone” morphology [101]. Some EPCs are selected based on their adherence properties and they grow as monolayers with morphology similar to endothelial cells but morphology alone is not a useful indicator to show actual differentiation into mature endothelial cells.

The ability to identify endothelial cells based on their increased metabolism of acetylated low density lipoprotein (Ac-LDL) was examined using the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) [102]. In this assay, ECs are highly fluorescent compared to pericytes and smooth muscle cells when incubated with the compound. This uptake assay has been used to demonstrate EC properties of EPC but the problem is that Ac-LDL is also taken up by monocytes and macrophages, making them phenotypically indistinguishable from EPC [95].

Lectins are carbohydrate-binding proteins of nonimmune origin that can bind to a wide variety of glycoproteins bearing the same carbohydrate motif and have been widely used

to interpret the sugar codes on glycoproteins [103]. A lectin called Ulex Europaeus Agglutinin Type I (UEA-1) has specific affinity for the 1-fucose moieties found on endothelial cells [104]. However, monocytes, fibroblasts, megakaryocytes and other blood cells bind to UEA-1 and thus UEA-1 binding cannot solely be used as a marker for EPC [95, 105, 106].

Tube formation assays are performed to assess the functional properties of endothelial cells. Many cells are capable of lining up to each other and coalesce on a two-dimensional (2D) surface to form a cord network, but only ECs undergo tube formation creating a lumen. This can only be observed in a three-dimensional (3D) system [107]. Many of the putative EPC form cords using a 2D matrix but have not been shown to form 3D tubes. Collagen and fibrin are components of the most widely used matrix in 3D assays that mimic components of the extracellular matrix to which EC are constantly exposed to in vivo. Other cell types typically do not form tubes in such matrices; thus, the endothelial potential of putative progenitors can be reliably tested in these 3D systems.

The de novo formation of functional vessels in vivo is the most rigorous test to determine whether a specific cell population can contribute to postnatal neovascularization [108]. This can be done with intravenous injection into injured animals, direct injection into injured tissues, and implantation of the cells of interest within a matrix or tumor environment followed by careful imaging analysis using a microscope capable of visualizing 3D vascular structures and identifying whole singe cells.

Once EPC have incorporated into host vessel in vivo, the gene/protein expression indicative of mature, functional EC must be determined to demonstrate that a phenotypic change has taken place to confirm vasculogenesis.

Several distinct population of circulating cells were found to be involved in repairing the endothelium: the colony forming unit-Hill (CFU-Hill), circulating angiogenic cells (CAC) and endothelial colony forming cells (ECFC). While CFU-Hill express some endothelial markers and form colonies in culture, they also highly express myeloid cell markers (CD14, CD45, CD115). The colonies are contaminated with hematopoietic progenitor cells and T lymphocytes, have poor proliferative potential and are capable of phagocytosis [94, 96, 109]. CACs are spindle-shaped, show the same antigenic characteristics as CFU-Hill cells, and are contaminated with platelets that possess surface antigens similar to endothelial cells [110, 111].

1.6.3 ENDOTHELIAL COLONY FORMING CELL (ECFC)

ECFCs, derived from adult and umbilical cord blood, are cells that appear much later in culture with colonies forming between 14 and 21 days and are also called outgrowth endothelial cell [112]. ECFC display characteristic cobblestone morphology, express endothelial but not myeloid markers, are highly proliferative, and do not ingest bacteria [94]. ECFC form networks in vitro and blood vessels de novo in vivo that inoculate with nearby host vessels and become part of the systemic circulation of the host animal [94, 113, 114]. They exhibit all the characteristic of a true progenitor cell and thus can aptly be called EPC. The differences between CFU-Hill, CAC and ECFC are shown in figure I-3 and table I-1.

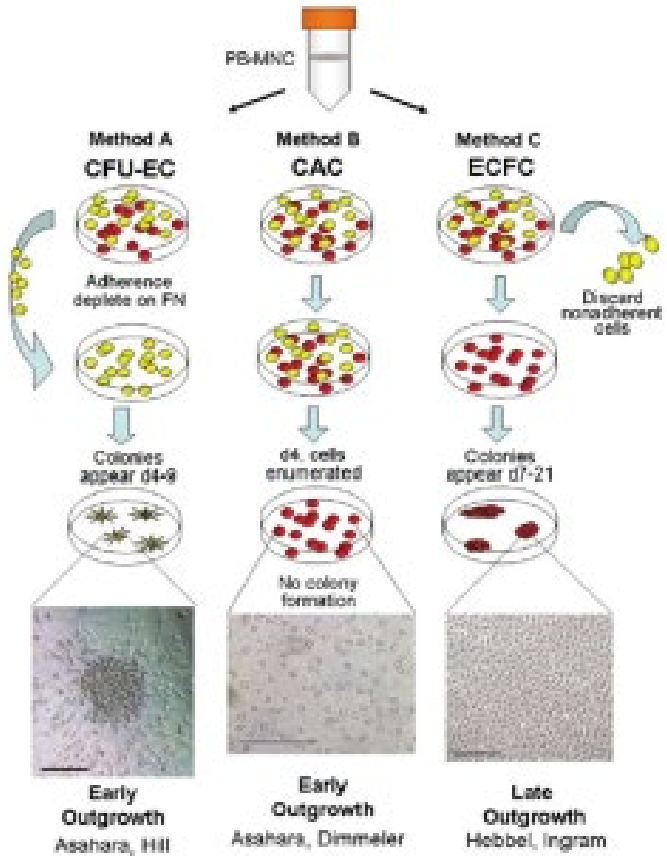


Figure I-3. Different EPC culture methods.

Culture of CFU-Hill, Method A, scale bar = 100 μm) is a 5-day process wherein non-adherent MNCs give rise to the EPC colony. CAC, Method B, scale bar = 200 μm) are the adherent mononuclear cells of a 4- to 7-day culture procedure. They typically do not display colony formation. ECFCs, Method B, scale bar = 400 μm) are derived from adherent MNCs cultured for 7–21 days in endothelial conditions and colonies display a cobblestone morphology. [Images were collected using a Zeiss Axiovert 2 inverted microscope with x10/0.25 Ph1 CP-ACROMAT (CFU-Hill), x32/0.40 Ph1 LD-ACROSTIGMAT (CAC), or x5 CP-ACHROMAT/0.12 Ph0 (ECFC) objectives. Images were acquired using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI, USA) with the manufacturer's software. Images cropped and scale bars added in

Adobe Photoshop version 8.0.] CFU-Hill = Colony-forming unit – Hill, MNC = mononuclear cell, EPC = endothelial progenitor cell, CAC = Circulating angiogenic cell, ECFC = Endothelial colony forming cell. Figure modified and adapted from Prater DN et al. (2007) [115].

	CFU-Hill	CAC	ECFC
Clonal proliferative status	-	-	+
Replicating ability	-	-	+
In vitro tube formation	±	±	+
In vivo de novo vessel formation	-	-	+
Homing to ischemic sites in vivo	+	+	+
Paracrine augmentation of angiogenesis	+	+	±
Phagocytosis of bacteria	+	+	-
Non-specific esterase expression	+	+	-
Phenotypic appearance	CD34±, CD133+, VEGFR2+, CD45±, CD14±, CD115+, CD31+, $ALDH^{bright}$, acLDL uptake, UEA-1 lectin binding,	CD34±, CD133+, VEGFR2+, CD45±, CD14±, CD115+, CD31+, $ALDH^{bright}$, acLDL uptake, UEA-1 lectin binding,	CD34+, CD133-, VEGFR2+, CD45-, CD14-, CD115-, CD31+, $ALDH^{bright/lo}$, acLDL uptake, UEA-1 lectin binding,

	eNOS, von Willibrand+	eNOS, von Willibrand+	eNOS, von Willibrand+

Table I-1. Characteristics of cells in the commonly used assays of "EPC" identification. Adherent cells that display the function are indicated by (+), those that do not display the function by (-), and if the literature provides conflicting evidence (±). Only the ECFC and progeny display the full properties one would attribute to an EPC. CFU-Hill = Colony-forming unit – Hill; CAC = Circulating angiogenic cell; ECFC = Endothelial colony forming cell; VEGFR2 = vascular endothelial growth factor receptor 2; ALDH = aldehyde dehydrogenase; UEA-1 = Ulex europeaus agglutinin-1; acLDL = acetylated low density lipoprotein; eNOS = endothelial nitric oxide synthase. Table modified and adapted from Hirschi et al. (2008) [116].

1.7 EPC IN RHEUMATIC DISEASE

The role of EPC in maintaining vascular homeostasis is a relevant topic in rheumatology because of the prominent vascular complications that is associated with chronic rheumatic inflammation. Altered peripheral blood numbers and altered function of EPCs have been described in patients with inflammatory rheumatic disorders like systemic sclerosis, SLE and vasculitides [117-119].

SLE has been associated with depleted [119-122], normal [52] or increased peripheral EPC numbers. Studies on SSc showed decreased peripheral EPC numbers in some studies [19, 123] but others found increased EPCs in the circulation compared to healthy controls [18, 117]. In dermatomyositis, circulating EPC were present in lower numbers and they displayed decreased ability to differentiate into mature EC [54]. There are conflicting reports regarding the presence and role of circulating EPC in SSc. Increased

numbers of EPC have been demonstrated in one study [124]; however in another the numbers were significantly reduced [19].

CHAPTER II

2.1 INTRODUCTION

Vascular dysfunction is widely reported to be present clinically and pathologically in patients with CIRD. Endothelial dysfunction occurs early in rheumatic disease and is associated with an increased risk of premature CVD over time. Both traditional and disease-specific risk factors contribute to the cardiovascular morbidity. There is good evidence that endothelial repair is impaired in vasculitis and this may contribute to vascular dysfunction and damage. EPCs have been identified as important players in maintaining vascular homeostasis. ECFC are endothelial cell precursors with clonal proliferative potential that display the ability to spontaneously form blood vessels upon implantation into host tissues, therefore are true EPCs that can be potential targets in understanding vasculitis or vasculopathy pathogenesis and recovery in the setting of autoimmune or rheumatic disease.

MPA is widely used off-label to treat many rheumatic conditions. The duration of MPA use is unpredictable but the majority of patients remain on this drug for several years or even decades. Despite good disease control, the higher cardiovascular risk of patients with rheumatic disease remains high. The frequent and chronic use of MPA in treating diseases associated with an increased risk for developing vascular dysfunction and consequent cardiovascular disease, raises the question as to the effects of MPA on ECFC number and function.

2.2 OBJECTIVES AND HYPOTHESIS

It is evident that the endothelium is not merely a target for injury, but rather, it is actively involved in the localization and propagation of inflammation and perhaps also the initiation of autoimmune responses. Patients with CIRD have significant endothelial

dysfunction which may be partly attributed to impaired endothelial repair from off-target effects of immunosuppressive drugs.

The purpose of this study is to determine the effect of MPA, a potent immunosuppressive drug commonly used in rheumatology, on blood vessel or circulating endothelial cell colony populations and their function.
The hypothesis is that MPA diminishes the proliferative potential and vasculogenic function of human ECFC.

The hypothesis was tested by:

- Determining the effects of MPA on ECFC proliferation, migration and vasculogenic function
- Determining the mechanisms by which MPA exerts effect on ECFC

2.3 MATERIALS AND METHODS

2.3.1 CULTURE OF HUMAN ECFC

Human umbilical cord blood samples from healthy term newborns, gestational age 37-40 weeks, were collected in CPD solution. Blood was diluted with Hanks balanced salt solution (HBSS, Invitrogen, Grand Island, NY) in equal parts. It was then layered on top of Histopaque 1077 (ICN, Costa Mesa, CA), centrifuged for 30 minutes at room temperature and washed with complete endothelial growth medium-2 (EGM-2) medium to isolate mononuclear cells (MNC). EGM-2 was made by combining endothelial basal medium (EBM, Cambrex, Walkersville, MD) and EGM™-2 BulletKit™ comprising of: human epidermal growth factor (hEGF), human basic fibroblast growth factor (hFGF-β), insulin-like growth factor (R^3-IGF-1), vascular endothelial growth factor (VEGF), heparin,

ascorbic acid and hydrocortisone, and supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT), 2% penicillin/streptomycin and 0.25 µg/mL amphotericin B. MNCs were re-suspended in EGM-2 medium and seeded onto six well plates precoated with type I rat tail collagen (BD Biosciences, Bedford, MA) and cultured in 37^{o}C with 5% CO_2 humidified incubator for 24 hours. Non-adherent cells and debris were aspirated and EGM-2 medium was added to each well. Medium was changed daily for seven days and then every other day until the first passage. Once confluent, cells were detached using TrypLE^{TM} Express (Gibco), counted and either plated onto 75-cm^2 tissue culture flasks pre-coated with type 1 rat tail collagen for further passage, or frozen at -80^{o}C for future use.

2.3.2 FREEZING AND THAWING OF ECFC

Confluent cells were detached from the plate and centrifuged at 220x g for 5 minutes. Supernatant was discarded and cells were re-suspended in complete EGM-2 with 10% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich) a density of 1x10^6 cells/ml cryovial. Cell vials were cooled in a Nalgene 5100 Cryo freezing container at -80°C. For longer term storage, cells were stored in liquid nitrogen.

Frozen cells were thawed at room temperature. Once almost defrosted, FBS was added and the solution transferred into a conical tube, centrifuged and re-suspended in complete EGM-2. Cells were transferred to tissue culture flasks or plates and allowed to adhere at least overnight. Cells were cultured as described above.

2.3.3 MPA

MPA stock solution was made by dissolving MPA powder ($C_{17}H_{20}O_6$, MW: 320.3; Selleck Chemicals) in 0.6% methanol before diluting in culture medium to the desired final concentrations.

2.3.4 GUANOSINE

Guanosine powder (Sigma-Aldrich) was dissolved in EGM-2 media to make 1000 μM concentration and then diluted to desired final concentration. Stock solution was made fresh with each experiment.

2.3.5 EFFECTS OF MPA ON HUMAN ECFC PROLIFERATION: CELL COUNTING BY TRYPAN BLUE EXCLUSION

Equal densities ($1x10^4$ cells) of ECFC were seeded onto 24-well culture plates and cultured with EGM-2 medium with or without MPA at 0.1, 1, 2.5 and 5 micromolar (μM) concentrations. Cells were detached, centrifuged and re-suspended in medium. An aliquot was counted in a hemacytometer in the presence of 0.4% Trypan blue dye (Thermo Fisher Scientific) at 1:2 dilution after 24, 48 and 72 hours of incubation. Viable cells were identified as Trypan excluding cells. Cell percentage was calculated by dividing the number of viable cells over total cells and multiplying by 100.

2.3.6 EFFECTS OF MPA ON HUMAN ECFC PROLIFERATION: SINGLE CELL ASSAY FOR CLONAL PROLIFERATIVE POTENTIAL

Single EC were sorted using a FACSAria Sorter (Becton Disckinson, San Jose) and placed into each well with complete EGM-2 medium in a 96-well tissue culture plate pre-coated with collagen. Wells were examined the day after plating to endure the presence of a single cell per well. Culture medium was replaced with complete EGM-2 medium

with or without MPA at various concentrations, and then incubated at 37°C with 5% CO_2 for 13 days with two media changes. On day 14, cells were fixed with 4% paraformaldehyde (PFA, Thermo Fisher Scientific) and stained with 4',6-diamidino-2-phenylindole stain (DAPI, Sigma-Aldrich). Each well was then examined for colony formation using a Zeiss Axiovert 25 CFL inverted microscope with a 10x CP-ACHROMAT/0.12 NA objective. For quantitative analysis, wells that had at least 2 EC were scored and examined further by visual counting. The colonies were classified as EC clusters (2 to 50 cells), low proliferative potential (LPP)-ECFC (51 to 2,000 cells), and high proliferative potential (HPP)-ECFC (more than 2,000 cells).

2.3.7 EFFECTS OF MPA ON HUMAN ECFC PROLIFERATION: CARBOXYFLUORESCEIN SUCCINIMIDYL ESTER (CFSE)

CFSE (Molecular Probes, Eugene, OR) lyophilized powder was dissolved in DMSO and stored as aliquots at -20°C. CFSE-DMSO stock solution was diluted in pre-warmed phosphate-buffered saline (PBS) to make a 5 μM concentration. Cultured ECFCs were trypsinized, centrifuged, re-suspended in PBS dye solution and incubated for 20 minutes at room temperature protected from light. 7.5-15x10^4 CFSE-stained cells were cultured in complete EGM-2 medium at defined concentrations of MPA at 0, 1, 3, 5 and 7 days. After appropriate time of culture, cells were harvested, fixed with 4% PFA, and stored at 4°C. On day 7, the CFSE mean fluorescence intensity (MFI) of all harvested samples was measured using flow cytometry (BD FACS Canto II) and analyzed using FlowJo software (TreeStar). To account for differences in CFSE staining between replicates, the MFI of the day 0 sample was used as an internal control. Thereby, the MFI of each sample was normalized by dividing it by the MFI of the day 0 control. Results are plotted on a log2 scale to visualize the doubling nature of cell proliferation. The data represent averages of 4 biological replicates.

2.3.8 EFFECTS OF MPA ON HUMAN ECFC MIGRATION: IN VITRO SCRATCH ASSAY

$4x10^4$ cells per well were plated in triplicate onto a 12-well plate and cultured in complete EGM-2 medium. Upon reaching 70-80% confluence, a single straight line through the cell monolayer was made by scraping the surface using a sterile p1000 pipette tip. Cells were washed once with PBS to remove cell debris and replaced with medium with or without MPA. The plates were placed in the incubator at 37°C for 6 to 24 hours. Images were taken at various time points after the scratch and we measured the average distance between the lines. Image J software (NCBI) was used to quantify gap distance.

2.3.9 EFFECTS OF MPA ON CAPILLARY NETWORK FORMATION: TWO-DIMENSIONAL (2D) MATRIGEL ASSAY

Growth-factor reduced Matrigel (BD Bioscience) was thawed at 4°C overnight. The gel was allowed to polymerize at 37°C for 30 minutes before use. EC were trypsinized and re-suspended in EGM-2 media. Cells were seeded at $1x10^4$ cells per well in triplicate onto 96-well plates coated with 50 µL of growth-factor reduced Matrigel. Complete EGM-2 medium with or without MPA were added to the well and incubated at 37°C with 5% CO_2 for 6 hours. Three images were taken from each well at 10x magnification using a Zeiss Axiovert 25 CFL inverted microscope with a 10x CP-ACHROMAT/0.12 NA objective. Total cord length and average cord area were calculated using ImageJ software (NCBI).

2.3.10 EFFECTS OF MPA ON CAPILLARY NETWORK FORMATION: THREE-DIMENSIONAL (3D) COLLAGEN ASSAY

Reagents for 3D collagen matrix were purchased from GeniPhys (Zionsville, IN) unless otherwise specified. To make the matrix, stock oligomer was diluted in 0.01 N hydrogen

chloride (HCl) and neutralized according to the manufacturer's recommendations to achieve a final oligomer concentration of 1.56 mg/ml (250 Pa matrix stiffness) before adding 10% human platelet lysate (SALK, Graz, Austria). Matrix was maintained in solution at 4°C until used. $1x10^5$ ECFCs (60 µL) were suspended in the collagen matrix. The collagen-cell suspensions were plated onto 96-well culture plate wells and allowed to polymerize at 37°C for 30 minutes before covering with MPA treated and control culture medium. Cells were incubated at 37°C with 5% CO_2 with daily medium change. The cultures were checked under a microscope every 24 hours until lumenized vessel-like structures were formed and identified. After three days, cells were fixed with 4% PFA and stained with 0.1% toluidine blue O dye (Thermo Fisher Scientific). Three images were taken at a uniform depth from the surface of the matrix using a Zeiss Axiovert 25 CFL inverted microscope. Average cord area was quantified using ImageJ software (NCBI).

2.3.11 CELL CYCLE ANALYSIS WITH 5'-BROMO-2'-DEOXYURIDINE (BrdU)

ECFC ($3\text{-}5x10^5$) were exposed to either vehicle control or MPA 1 µM. After 3 days, cells were treated with BrdU labeling reagent (Invitrogen) for 1 hour at 37°C with 5% CO_2. Cells were stained using Alexa Flour 488 mouse anti-BrdU (Invitrogen) for 90 minutes at room temperature and 7-AAD (Life Technologies) for 15 minutes at room temperature. Samples were analyzed by flow cytometry on the LSRII 407nm laser and a minimum of 10,000 events were recorded per sample. The percentage of cells at the G0/G1, S and G2/M phases of the cell cycle were analyzed using FlowJo Single Cell Analysis Software vX.0.6.

2.3.12 APOPTOSIS

The effect of MPA on ECFC death by apoptosis was examined using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) analysis by flow cytometry. 5-35x10^4 cells were plated and treated with varying concentrations of MPA for 7 days with one media change. Cells were collected and incubated for 15 minutes at room temperature with annexin V-FITC (BD Pharmigen) and PI (BD Pharmigen) in the dark, and immediately analyzed on a flow cytometer (BD Biosciences San Jose, CA). Data were processed using FlowJo software (TreeStar), determining the percentage of apoptotic cells.

2.3.13 SENESCENCE: CELL MORPHOLOGY

7.5x10^5 cells were plated on T75 tissue culture flask and allowed to attach overnight. Cells were then exposed to complete EGM-2 with or without MPA. Images were taken after 3 days.

2.3.14 SENESCENCE: SENESCENCE- ASSOCIATED BETA-GALACTOSIDASE (SA-β-GAL) STAINING

To determine MPA effect on cellular senescence, SA-β-gal assay was performed using a Senescence Detection kit (BioVision, Milpitas, CA) according to the manufacturer's instructions. Upon senescence, the lysosomal mass is increased, leading to production of higher levels of β-galactosidase [125]. Cells were washed twice with PBS, fixed using the supplied fixing solution for 10-15 minutes, washed again with PBS, stained with SA-β-gal solution (X-gal in dimethylfomamide 20 mg/ml plus staining supplement) and incubated overnight at 37^0C without CO_2 protected from light. After 24 hours, staining solution was discarded and the cells were overlaid with 70% glycerol. To quantify SA-

beta-gal activity, the percent of positively stained blue cells (senescent cells) versus total cells were counted (500-600 cells).

2.3.15 SENESCENCE: GENE MARKERS

ECFCs were grown to 70-80% confluence in T75 tissue culture flasks after thawing. The media was removed, cells washed in PBS, trypsinized and re-suspended in complete EGM-2 culture medium. Equal density of cells was plated on 6-well tissue culture plates and incubated at 37°C overnight to allow cell attachment. Media was removed, washed in PBS once, replaced with EGM-2 media with or without MPA and incubated for 3 days.

2.3.15.1 ISOLATION OF RNA

RNA was isolated from ECFC to examine the effect of MPA on expression of genes that are associated with senescence. Total mRNA was extracted from the cell using RNAeasy Micro Kit (Qiagen). All reagents and plastic-ware used in the isolation of RNA were molecular grade and RNAse-free. The concentration and purity of the extracted RNA was measured using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE).

2.3.15.2 REVERSE TRANSCRIPTION

Complementary DNA (cDNA) was synthesized from RNA by reverse transcription and used as a template for PCR amplification performed on a LifeEco thermal cycler. 500 ug of total RNA was reverse transcribed using SuperScript™ III First-Strand Synthesis System (Invitrogen). Each sample was incubated with Oligo-dT primers and 10mM of dNTP mix at 65°C for 5 minutes, then placed on ice for at least 1 minute. To each tube, 2µl dithiothreitol (DTT) (100mM), 4µl $MgCl_2$ (25mM), 2µl 10x buffer, 1µl RNaseOUT (40 U/µL) enzyme and 1µL of SuperScript III RT (200 U/µL) enzyme were added. The extension step was carried out at 50°C for 50 minutes and the reaction terminated at

85°C for 5 minutes. Then 1 µL of RNase H was added and incubated at 37°C for 20 minutes. cDNA synthesis reaction was used immediately for polymerase chain reaction or stored at -20°C until used.

2.3.15.3 REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

qRT-PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems, UK) and real time detection on 7500 Real-Time PCR machine (Applied Biosystems, UK). Each 10 µL PCR reaction was carried out in triplicate, and contained 1 µL cDNA, SYBR Green and the appropriate forward and reverse primers (5nM each). PCR was carried out according to the following protocol: 95°C for 10 minutes then 40 cycles of denaturation (95°C for 15 seconds), annealing (60°C for 60 seconds) and extension (60°C for 60 seconds).

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to beta-2-microglobulin mRNA expression levels.

ΔCt: ratio = $2^{Ct\ (GOI) - Ct\ (Ref\ gene)}$

ΔΔCt: ratio = $2^{-\Delta Ct\ (sample\ of\ interest) - \Delta Ct\ (\ control\ sample)}$

The following primers (Table II-1) were used:

Gene	Sequence (5'->3')
Human p16^{INK4a}	Forward: GGGGGCACCAGAGGCAGT
	Reverse: GGTTGTGGCGGGGGCAGTT
Human p21^{WAF}	Forward: AGTCAGTTCCTTGTGGAGCC
	Reverse: GACATGGCGCCTCCTCTG
Human p27^{Kip1}	Forward: TAATTGGGGCTCCGGCTAAC

	Reverse: GAAGAATCGTCGGTTGCAGGT
Human β-2-microglobulin	Forward: CTCCGTGGCCTTAGCTGTG
	Reverse: TTTGGAGTACGCTGGATAGCCT

Table II-1. List of primers

2.3.16 SENESCENCE: p53 PROTEIN LEVEL

2.3.16.1 CELL LYSATE PREPARATION

Cells were cultured with and without MPA for 3 days. After removing the media, plates were washed with ice cold PBS and cells were scraped into cell lysis buffer mix. Tubes containing the lysate buffer mixture were placed on a shaker at 300 rpm for 10 minutes at room temperature. Total protein concentration was determined by bicinchoninic acid (BCA) method.

2.3.16.2 ELISA

p53 (Total/Phospho) InstantOne ELISA Kit (Affymetrix) was used to specifically detect endogenous levels of total (tot) and phosphorylated (ph) p53 protein generation according to the manufacturer's instructions. 0.2-0.3 mg/ml protein lysates were added to each assay well followed by prepared tot-p53 and ph-p53 antibody cocktail (capture antibody and detection antibody regents). After a 1 hour incubation at 300 rpm, wells were washed 3x with wash buffer before adding detection reagent. The reaction was terminated with stop solution after 15 minutes incubation with shaking at 300 rpm and the absorbance immediately measured spectrophotometrically at 450 nm.

2.3.17 GUANOSINE AND MPA

The consequence of IMPDH inhibition by MPA is a reduction in guanine nucleotide pools. We tested whether MPA exerts its effect on ECFC IMPDH enzyme activity by measuring whether the addition of guanosine can reverse the suppressive cell growth effects of MPA. The following experiments were performed as described above: cell counting by trypan blue exclusion, senescence gene markers and p53 ELISA.

2.3.18 STATISTICAL ANALYSIS

Unless otherwise mentioned, all experiments were performed at least three times with 2-3 technical replicates for each assay. Data are presented as the means±SD. Two-tailed Student's *t*-test or Fisher's exact test were used to assess for significance of differences between two groups. All statistical analyses were implemented using Graphpad Prism or Microsoft Excel software.

2.4 RESULTS

2.4.1 MPA INHIBITS ECFC PROLIFERATION

To determine whether endothelial cells are affected by MPA, human cord blood derived ECFCs were cultured with increasing MPA concentrations through the usual patient targeted therapeutic range and examined for cell proliferation. The cell number increased at least 4-fold after 48 hours and 10-fold after 72 hours of culture in untreated control cells and cells exposed to 0.1 μM MPA concentration. There was no significant change in viable cell counts of ECFC cultured in 1, 2.5 and 5 μM MPA concentrations at either timepoint (Figure II-1.), suggesting a complete block in cell proliferation.

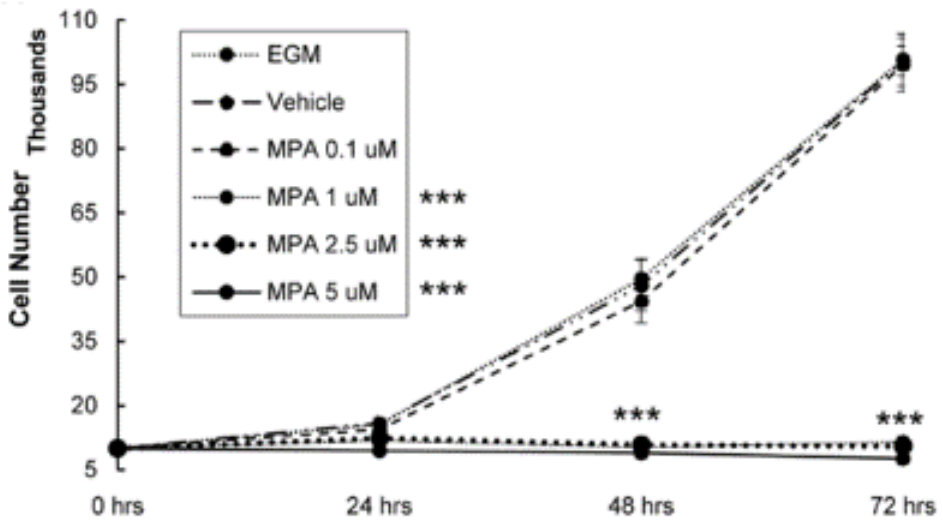


Figure II-1. ECFC number after exposure to MPA measured by cell counting.

Cell growth curve was evaluated using trypan blue staining to measure viable cells at 24, 48 and 72 hours. 1-5 μM concentration of MPA inhibited cell proliferation (n = 3). Results represent the mean ± SD.*** $P < 0.001$ compared to vehicle. MPA = mycophenolic acid

ECFC display a hierarchy of proliferative potential that can be discriminated on a single-cell level [112]. Single cell assay was performed to evaluate the effect of MPA on ECFC clonogenic potential. MPA concentrations (0.1-1 μM) were selected based on results from the cell proliferation assay with Trypan blue staining. At 0.1 μM MPA concentration, 89.5% (±2.0%, NS) of the single endothelial cells underwent ≥1 cell division. We observed that 40.1% (±8.2%, NS) of the total cells that divided had formed colonies of 51-2,000 cells per well (LPP-ECFC) and 55.7% (±16%, NS) formed >2,000 cells (HPP-ECFC that display self-renewal potential). At 1 μM MPA concentration, 70% (±8%, $p<0.05$) of the single cells plated divided and the majority of cells were restricted to forming small EC clusters (92.9%±3.5%, $p<0.005$). No HPP-ECFCs were seen to emerge from either 0.56 μM or 1 μM MPA treated cells. MPA treatment resulted in a dose-dependent decrease in overall colony formation (Figure II-2). The loss of HPP-ECFC at the higher doses suggests that the most primitive cells are more susceptible to MPA effects.

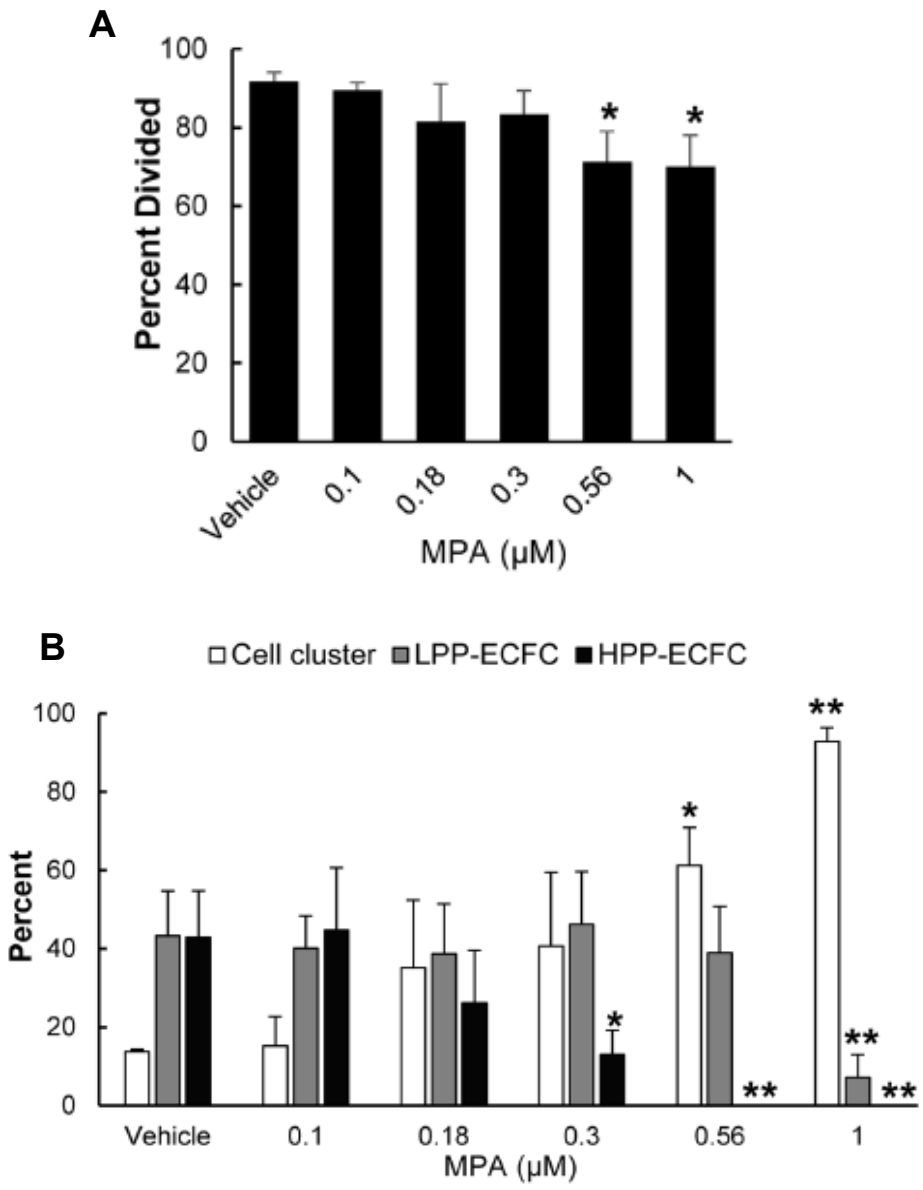


Figure II-2. Clonogenic potential ECFC following MPA treatment.

(A) Percentage of single cord blood-derived ECFC undergoing at least one cell division 14 days after MPA treatment (n = 3). (B) Percentage of cell clusters, LPP-ECFCs and HPP-ECFC 14 days after MPA treatment using single cell analysis (n = 3). Results represent the mean ± SD. *P <0.05, **P<0.005 compared to vehicle. ECFC = endothelial colony forming cell, EGM = endothelial growth medium, MPA = mycophenolic acid, LPP-

ECFC = low proliferative potential – endothelial colony forming cells, HPP-ECFC = high proliferative potential-endothelial colony forming cells.

ECFC proliferation was measured using CFSE staining. The CFSE dye stains intracellular proteins and is evenly distributed in dividing progeny cells after cellular division. As such, the rate of decrease of CFSE MFI among cultured cells is proportional to their rate of proliferation. We used flow cytometry to measure the MFI of CFSE-stained ECFC during 7 days of culture at increasing doses of MPA. As expected, cells cultured with vehicle or 0.1 μM MPA regularly divided and displayed diminished MFI over the 7 days culture. We observed significantly greater CFSE MFI retention (indicative of lack of cell division) in ECFC treated with MPA 0.5 and 1 μM MPA compared with ECFC treated with vehicle control (Fig II-3).

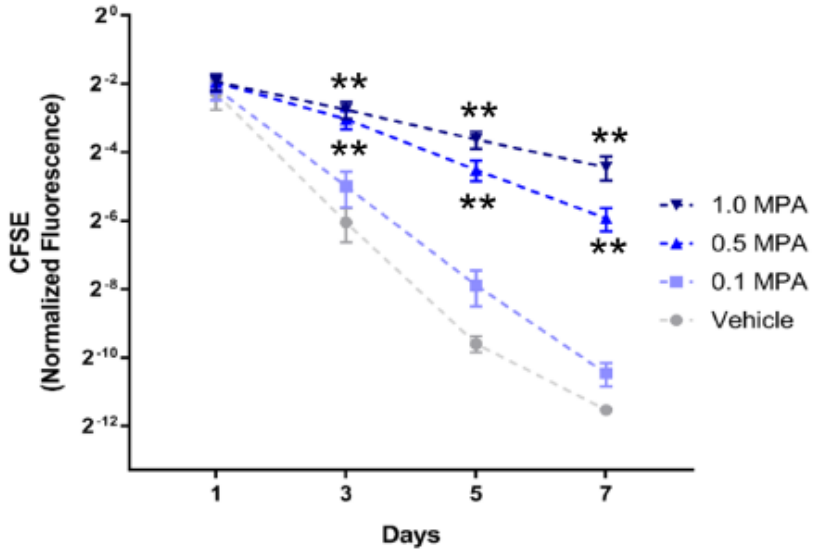


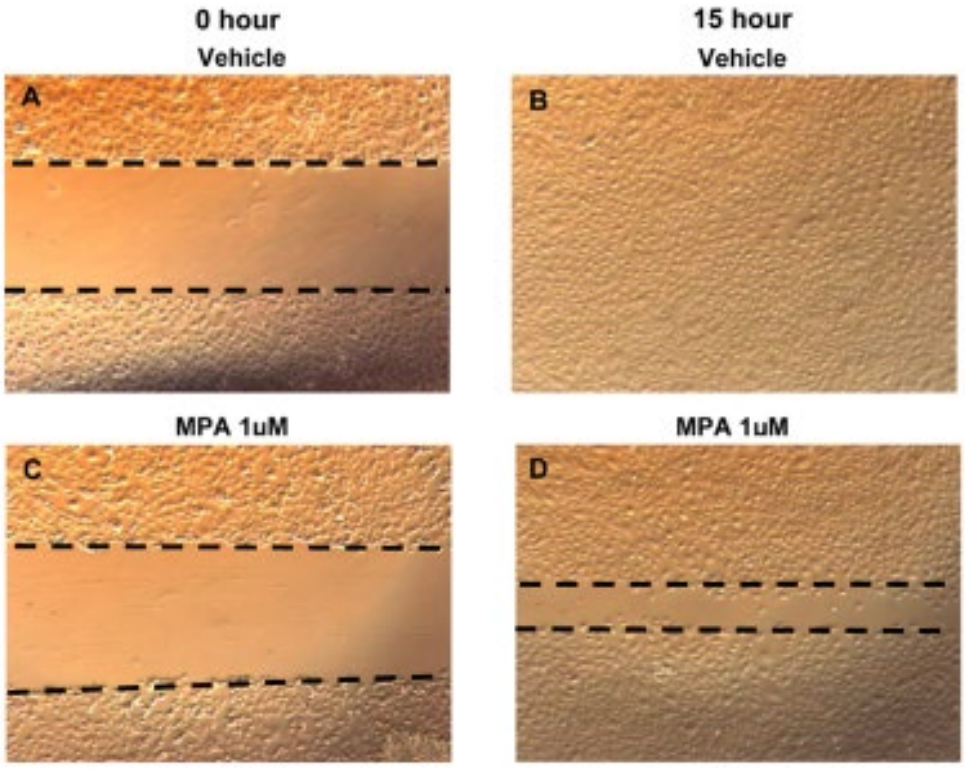
Figure II-3. Effect of MPA on ECFC proliferation measured by CFSE.

Proliferation of ECFC in the absence or presence of MPA (0.1, 0.5 and 1 μM) measured after 1, 3, 5 and 7 days by a FACS-based CFSE dilution assay. While 0.1 μM MPA did not impact ECFC division, MPA at higher concentrations significantly diminished ECFC division (n = 4). Results represent the mean ± SD. **P<0.005 compared to vehicle.

CFSE = carboxyfluorescein succinimidyl ester, MPA = mycophenolic acid

2.4.2 MPA DELAYS ECFC MIGRATION

ECFC migration into a denuded vessel is a vital process in wound healing. To determine whether MPA inhibits migration of ECFC, we performed an *in vitro* scratch assay. A line was drawn along the leading edges of each cell front at time 0 hour and 15 hour and we measured the gap distance as percentage of un-occupied area at 15 hour divided by 0 hour exposure to media with and without treatment. Untreated cells completely closed the gap when examined after 15 hours, but a cell-free area is clearly visible in the MPA-treated (1 μM) cells (Figure II-4). The lag in gap closure observed after drug treatment can be attributed to delayed cell migration.



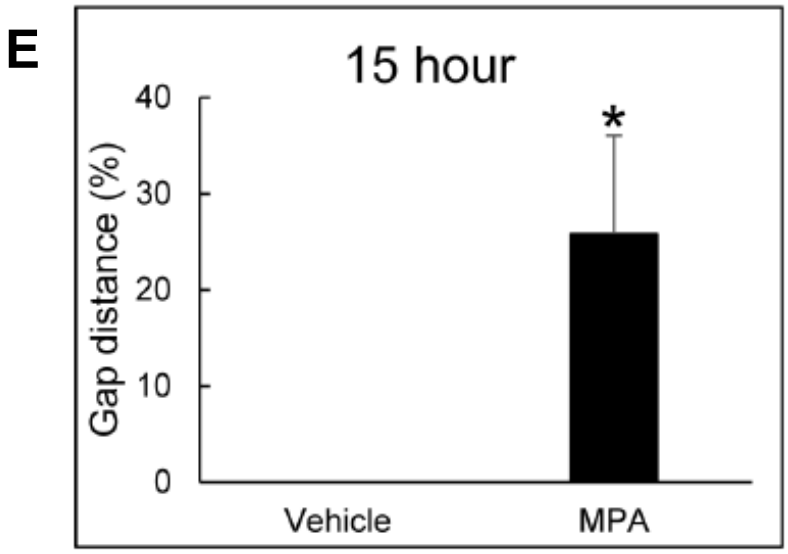


Figure II-4. MPA treatment impairs ECFC migration.

Cell migration was assessed using an *in vitro* scratch assay with and without MPA treatment. Representative phase-contrast images of ECFC incubated with vehicle control media at time 0 hour after wounding (A) and closure of the gap after 15 hours (B). ECFC incubated with 1 μM MPA at time 0 hour after wounding (C) and presence of a gap after 15 hours (D). Quantification of gap distance after 15 hours of incubation (E). Gap fully closed in vehicle treated wells while a gap is still clearly visible in cells treated with 1 μM MPA (25.8% ± 10%, *p<0.005) compared to vehicle (n = 4 to 6). MPA = mycophenolic acid, ECFC = endothelial colony forming cells

2.4.3 MPA REDUCES ECFC FUNCTION

Normal EC reparative function is also important in maintaining adequate vascular homeostasis. In the 2D assay, cells align with each other and form cord networks. The addition of MPA 2.5 μM concentration significantly reduced average cord area and total cord length by 29% (0.082 mm^2±0.009, P = 0.037) and 54% (5.45 mm^2±0.75, P = 0.026) respectively, compared to untreated cells (Figure II-5). In the 3D assay, single cells are suspended in gel and form vascular structures that emerge through a process of

cytoplasmic vacuolation [126]. The 3D assay is thus likely to be more representative of vessel formation *in vivo* than the 2D assay. The capillary-like structures with patent lumens were measured. Treatment with 1 μM MPA reduced tube formation to very rudimentary tubular structures. The average vascular area of cells exposed to MPA 1 μM and 2.5 μM concentrations was significantly smaller than the untreated cells by 78% (0.017 mm^2±0.004, P = 0.0006) and 88% (0.009 mm^2±0.0006, P = 0.003) respectively, compared to the untreated cells (Figure II-6). These data suggest that MPA inhibits the functional vasculogenic properties of ECFC.

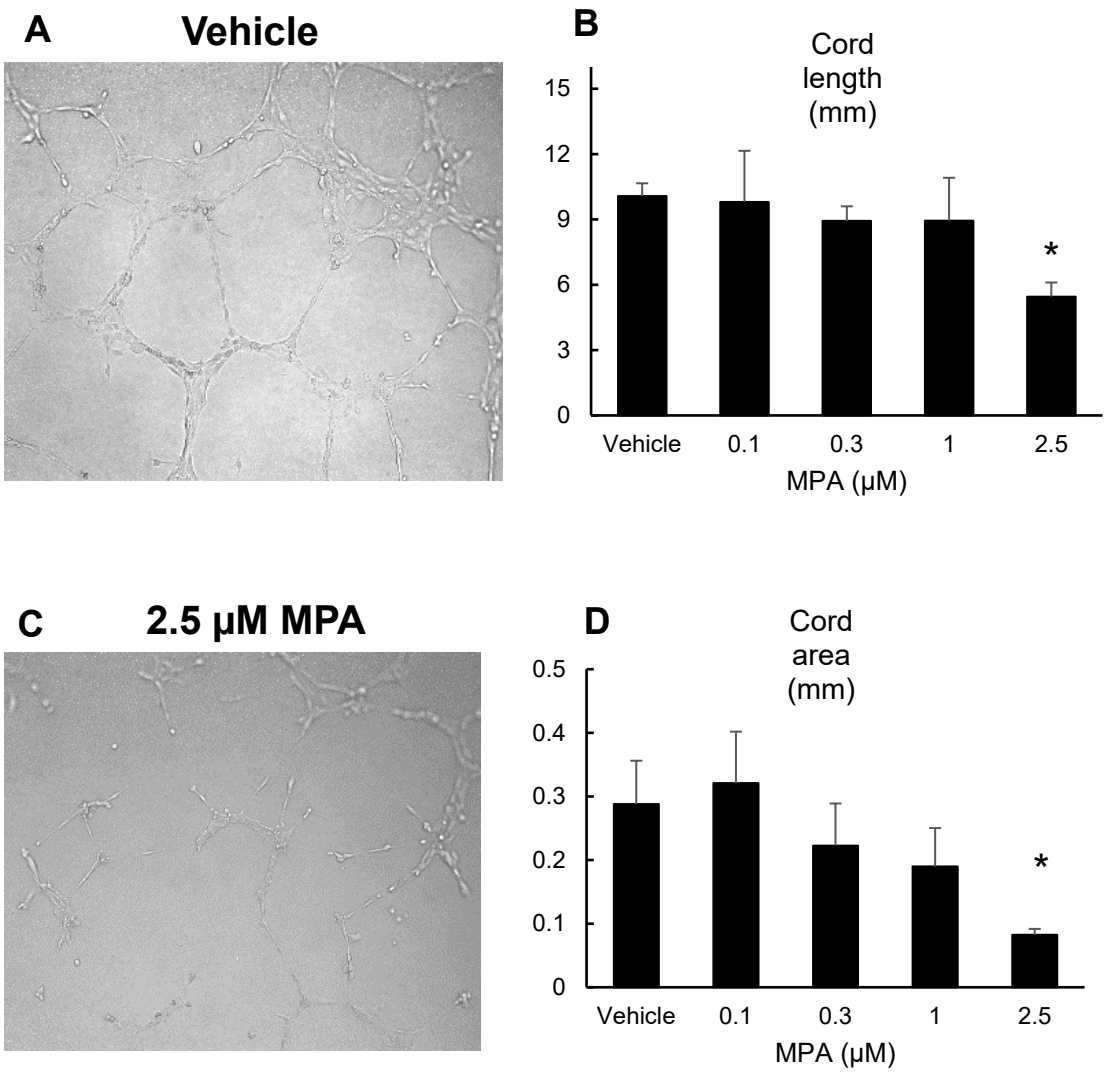


Figure II-5. Diminished ECFC vasculogenic function after MPA treatment (2D).

Representative photomicrographs (magnification, x10) of ECFC following no treatment (vehicle) or treatment with 2.5 μM MPA in 2D assay (A). 2D Matrigel assay showed that there is decreased average total cord length (B) and cord area after MPA treatment. *P <0.05 compared to vehicle (n=3). ECFC = endothelial colony forming cells, 2D = two-dimensional, MPA = mycophenolic acid, mm = millimeter.

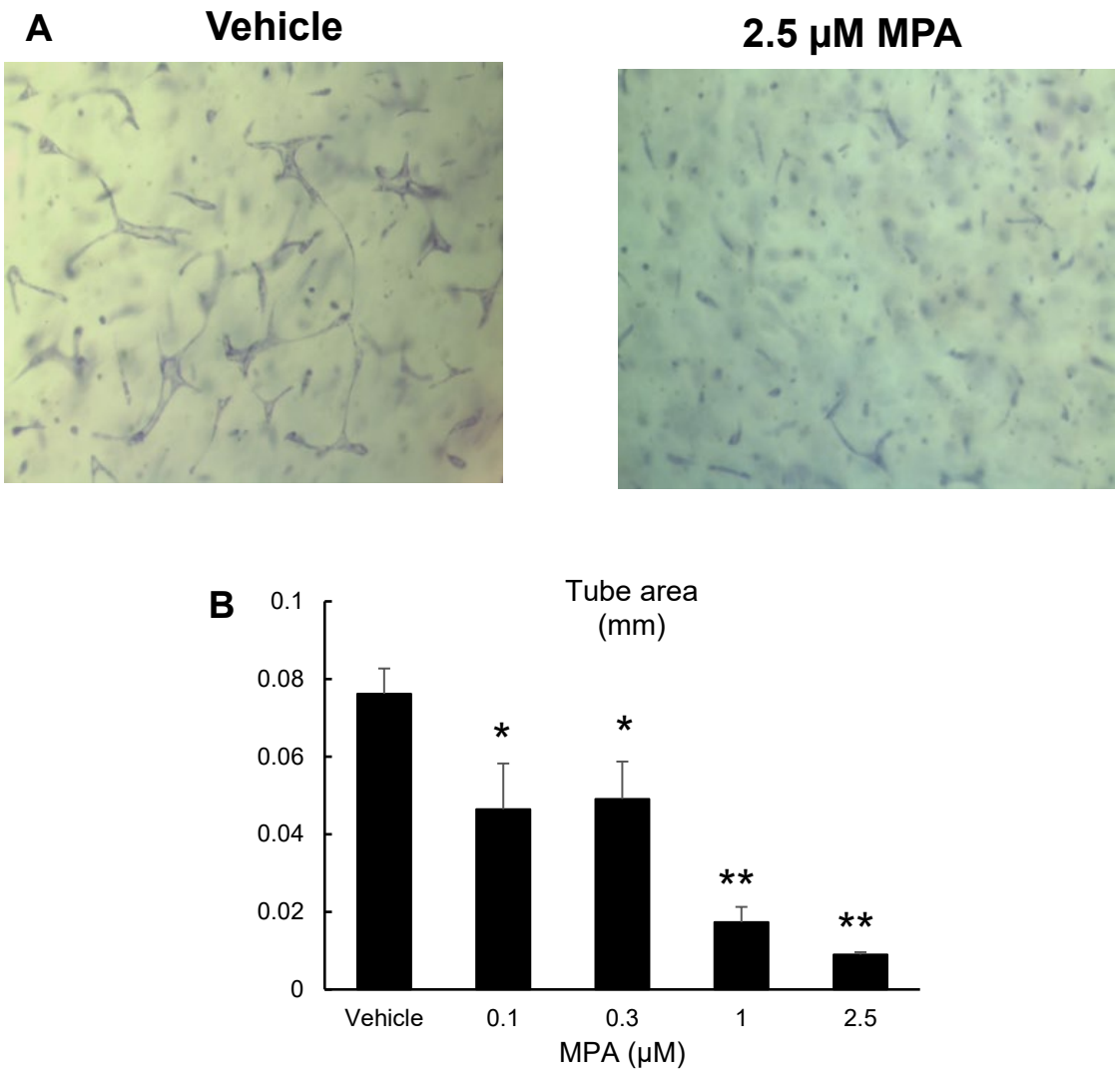


Figure II-6. Diminished ECFC vasculogenic function after MPA treatment (3D).

Representative photomicrographs (magnification, x10) of ECFC following no treatment (vehicle) or treatment with 2.5 μM MPA in 3D assay (A). 3D collagen assay showed decreased total vessel area after MPA treatment (B). *P <0.05, **P <0.005 compared to vehicle (n=3). ECFC = endothelial colony forming cells, 3D = three-dimensional, MPA = mycophenolic acid, mm = millimeter

2.4.4 MPA CAUSES ECFC CELL CYCLE ARREST

ECFCs were incubated with 1 μM MPA or vehicle. The effect of this treatment on progression through cell cycle analysis was analyzed using flow cytometry. MPA treatment led to 40-50% increase in the number of cells in the G1 phase, and the frequency of cells in the S phase fell by more than 50% (Figure II-7).

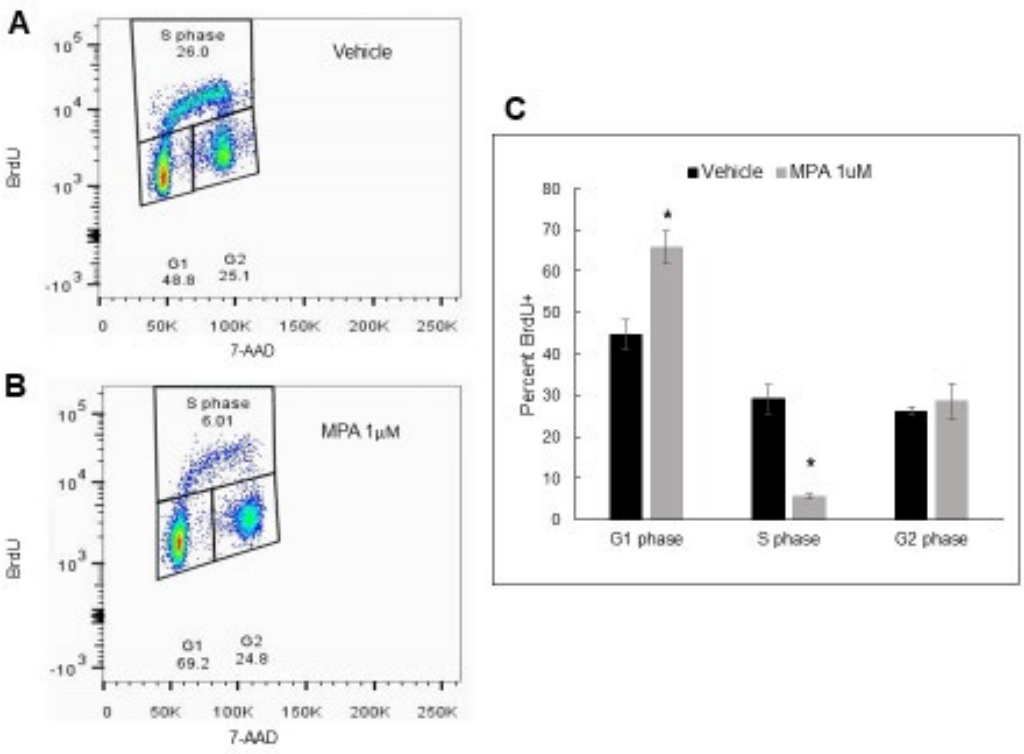


Figure II-7. Cell cycle analysis using BrdU and 7-AAD staining

Representative flow cytometry gating strategy to measure cell cycle progression of ECFC without (A) and with (B) MPA treatment. Quantitation of cell cycle analysis using flow cytometry data (n=3). *P<0.01. Abbreviations: MPA = mycophenolic acid

2.4.5 MPA DOES NOT CAUSE ECFC APOPTOSIS

To investigate whether the effect observed on cell proliferation was due to cell death, an apoptosis assay using flow cytometry was performed. The percentage of annexin V-negative/PI-negative (viable cells), annexin V-positive (undergoing early death) and PI-positive (dead or necrotic cells) cells cultured with or without 1 μM MPA were not statistically different (Fig II-8). This result indicates that the inhibition of proliferation after MPA treatment was not due to enhanced cell death.

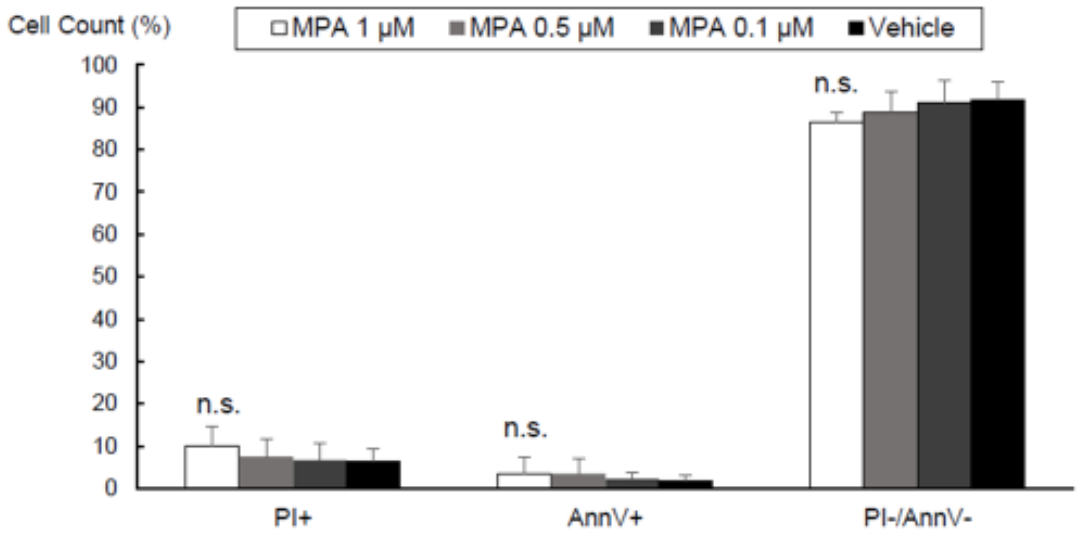


Figure II-8. No effect on apoptosis after adding MPA

Flow cytometric analysis of cells labelled with Annexin-V–PI double staining showing the number of viable (PI-/AnnV-), early death (AnnV+) and apoptotic (PI+) cells. Results

expressed as means ± SD (n=3). MPA = mycophenolic acid, PI = propidium iodide, AnnV = annexin V, n.s. = not significant

2.4.6 MPA INDUCES PREMATURE ECFC SENESCENCE VIA p21/p53 PATHWAY

Senescence is another mechanism by which cell growth and proliferation is inhibited. In order to determine whether MPA induces senescence in ECFC, SA-β-gal staining was used. Several human cells express β-galactosidase, histochemically detectable at pH 6, upon senescence in culture. This marker is expressed by senescent, but not presenescent, quiescent and terminally differentiated cells [125]. There is a significant dose-dependent increase in SA-β-gal positive cells (8-fold and 12-fold after 3 days of treatment with 0.5 μM and 1 μM MPA, respectively). This effect was more pronounced after 5 days of MPA treatment (Figure II-9).

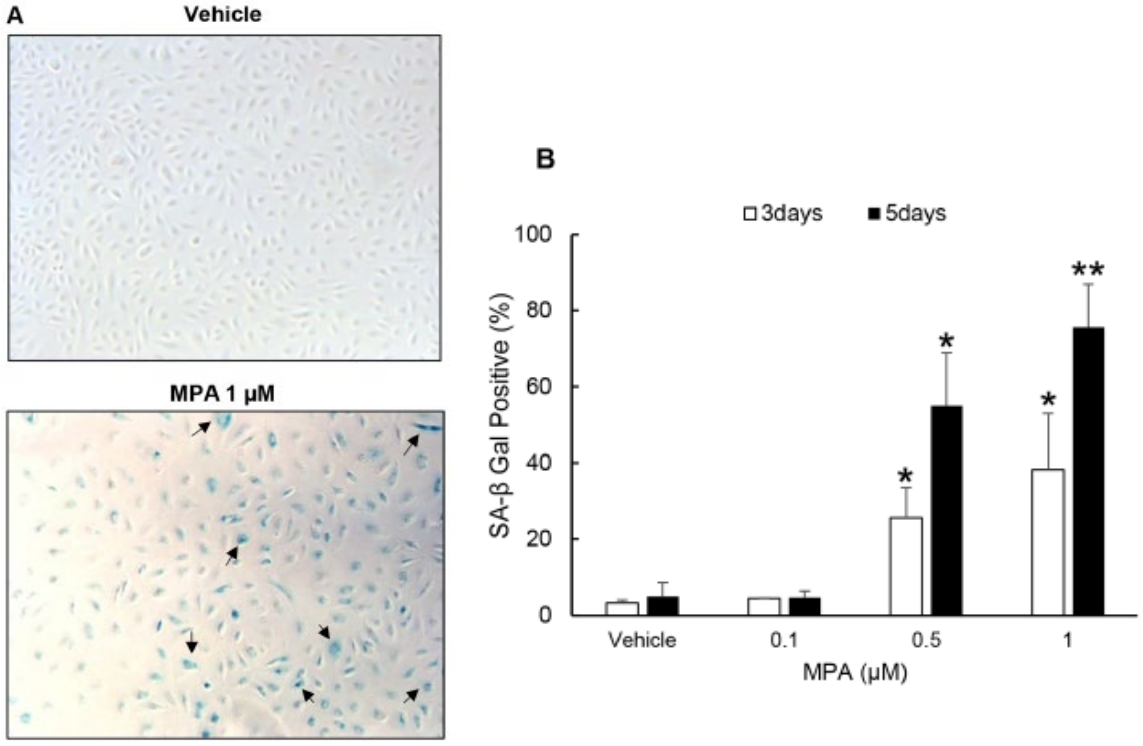


Figure II-9. MPA treated cells have increased SA-β-gal staining

(A) Photomicrograph of SA-β-gal staining of ECFC cultured with vehicle and 1 μM for 5 days. Blue color (arrow) indicates cellular senescence. (B) Quantification of SA-β-gal staining. At least 500 cells per individual sample were counted. The number of SA-β-Gal-positive cells treated with 0.5 and 1μM MPA concentrations was significantly greater than untreated cells. *P <0.05 compared to vehicle (n=3). SA-β-gal = senescence associated-beta galactosidase, MPA = mycophenolic acid.

To identify the mechanism by which MPA enhances susceptibility of ECFC to undergo senescence, we determined the expression of $p16^{INK4a}$, $p21^{WAF}$and $p27^{Kip1}$, cyclin dependent kinase (CDK) inhibitors and known senescence-associated inducers of cell cycle arrest. The expression of $p21^{WAF}$ in cells exposed to 1 μM MPA (11.45±1.84) increased significantly compared to untreated cells (6.77±2.67), but no significant difference in $p16^{INK4a}$ and $p27^{Kip1}$ expression (Figure II-10) was found in these groups.

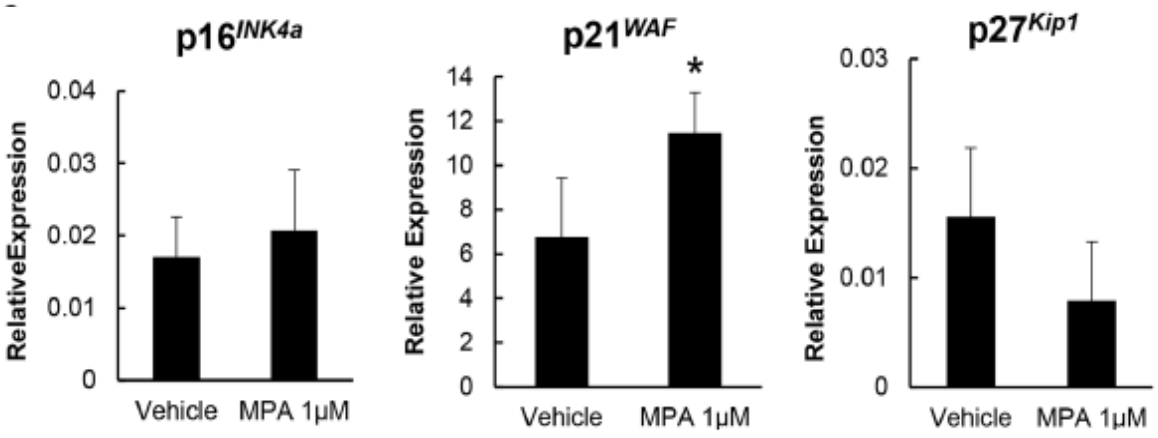


Figure II-10. MPA treated cells have higher senescence related $p21^{WAF}$ gene expression.

Gene expression of $p16^{INK4A}$, $p21^{WAF}$ and $p27^{Kip1}$ in untreated and treated cells normalized to B2M products. Results represent the mean ± SD compared (n = 3). *P < 0.05 relative to vehicle control. MPA =mycophenolic acid, B2M = beta-2-microglobulin

Since p53 directly induces $p21^{WAF}$ and is a key regulatory component of stress-inducible cell growth arrest, we assessed whether MPA-treated cells displayed increased p53 activation. By comparing the ratio of UV absorbance between phosphorylated (ph)-p53 and total (tot)-p53. A significantly higher level of p53 phosphorylation (activated state) was observed in the MPA treated cells compared to controls (Figure II-11).

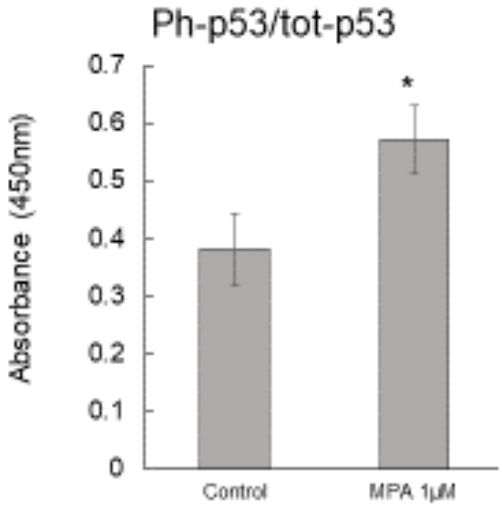


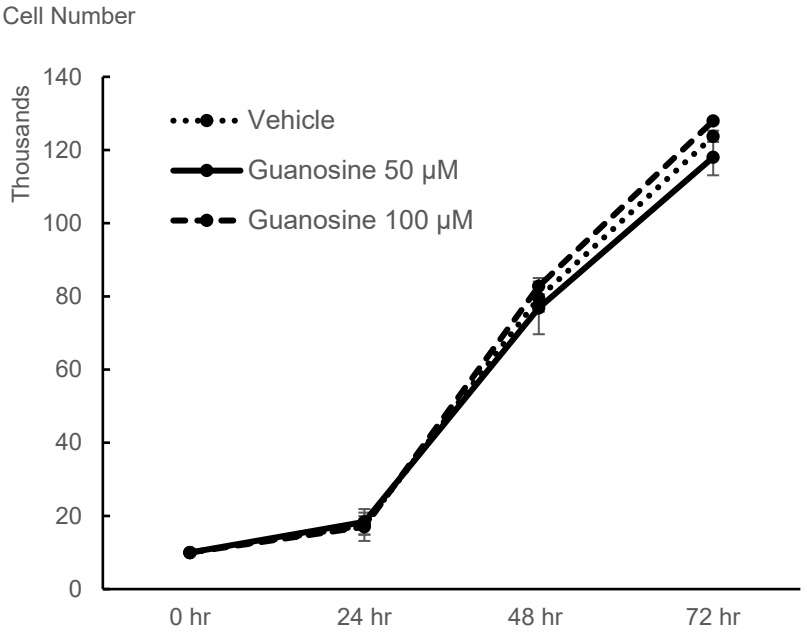
Figure II-11. MPA treated cells have higher phosphorylated p53 expression. Phosphorylation of p53 normalized to total p53 in cell lysates determined using ELISA method. Results represent the mean ± SD compared (n = 3). *P < 0.05 relative to vehicle control. OD = optical density, ph-p53 = phosphorylated p53, tot-p53 = total p53, MPA = mycophenolic acid.

These results show that MPA-treated cells become highly senescent, and imply that $p21^{WAF}$ gene upregulation is dependent on p53 activation to drive ECFC into senescence as a result of MPA treatment.

2.4.7 GUANOSINE RESTORES ECFC PROLIFERATION AND PREVENTS ECFC SENESCENCE

The consequence of IMPDH inhibition by MPA is a reduction in guanine nucleotide pools [127]. We tested whether MPA exerts its effect on ECFC IMPDH enzyme activity by measuring whether the addition of guanosine can reverse the suppressive cell growth effects of MPA. Guanosine at 50 or 100 μM alone was neither mitogenic nor inhibited cell growth. 100 μM guanosine together with 1μM MPA completely restored ECFC proliferation after 48 hours and 72 hours. We observed similar effects in cells exposed to medium containing 50 μM guanosine and 1 μM MPA but cell proliferation rescue was 17% lower (n.s.) than 100 μM of guanosine (Figure II-12). Thus, guanosine supplementation was sufficient to rescue ECFC from the anti-proliferative effects of MPA in a dose dependent fashion, consistent with the known mechanism of action of MPA on cells.

A


Cell Number
Thousands
140
120
100
80
60
40
20
0
Vehicle
Guanosine 50 μM
Guanosine 100 μM
0 hr
24 hr
48 hr
72 hr

B

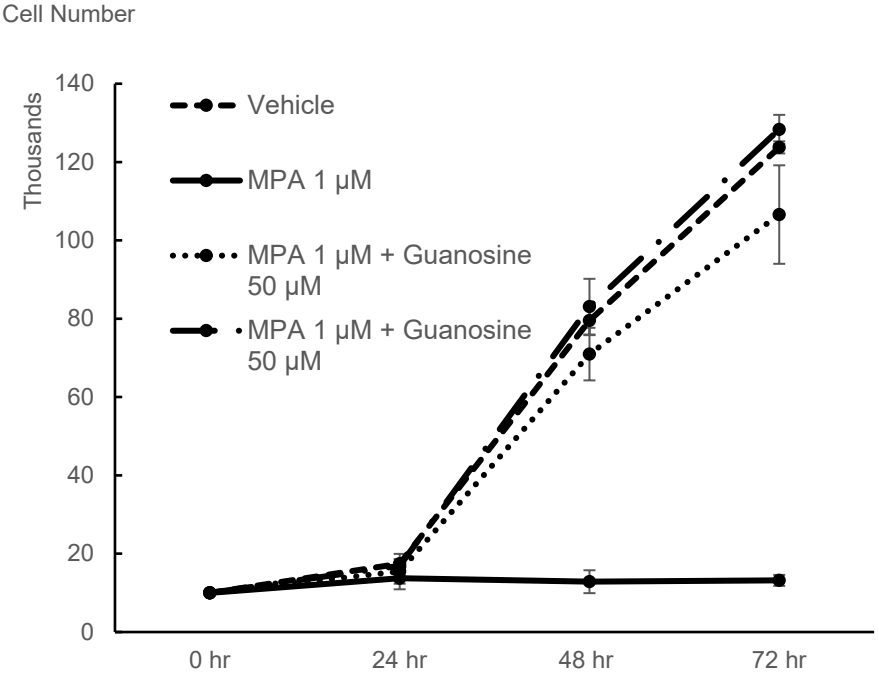

Cell Number
Thousands
140
120
100
80
60
40
20
0
Vehicle
MPA 1 μM
MPA 1 μM + Guanosine 50 μM
MPA 1 μM + Guanosine 50 μM
0 hr
24 hr
48 hr
72 hr

Figure II-12. Guanosine reversed MPA's inhibitory effect on cell proliferation.

Cell proliferation using **the** trypan blue exclusion assay shows that guanosine is not cytostatic or inhibitory (A), and that addition of guanosine rescues ECFC proliferation in a dose dependent manner (B). Results represent the mean ± SD compared (n = 3). ***P <0.001 relative to vehicle. MPA = mycophenolic acid.

Based on the ability of guanosine to rescue ECFC proliferation, we tested whether guanosine would rescue the cells from senescence. The addition of 100 μM guanosine decreased $p21^{WAF}$ gene expression to levels that were similar to those seen in control cells (Figure II-13).

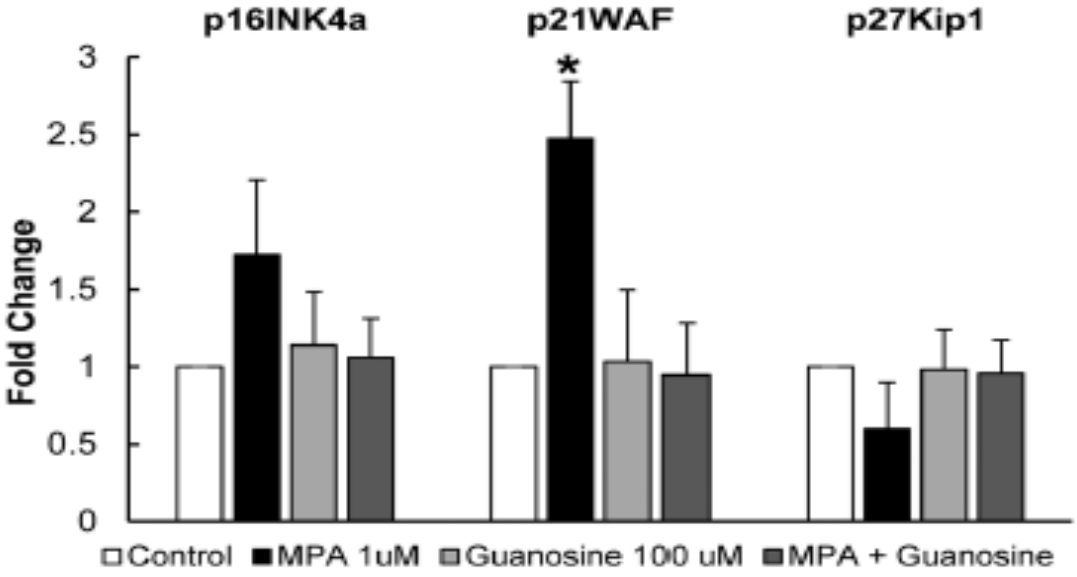


Figure II-13. Guanosine reversed MPA's inhibitory effect on senescence gene expression.

Relative fold expression of senescence associated gene markers $p16^{INK4a}$, $p21^{WAF}$ and $p27^{KiP1}$. Results represent the mean ± SD compared (n =3). *P < 0.05 relative to vehicle. MPA = mycophenolic acid.

Accordingly, guanosine completely restored p53 activation to control levels despite the presence of 1μM MPA (Figure II-14).

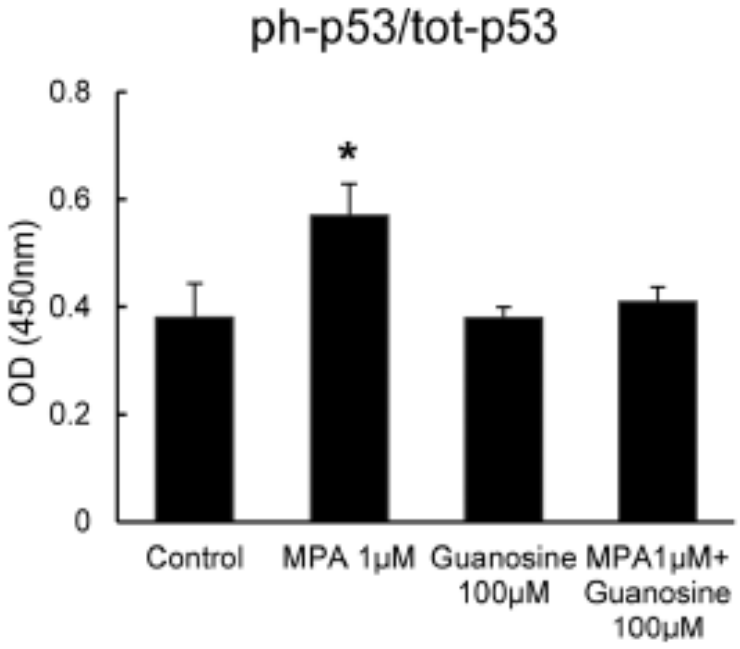


Figure II-14. Guanosine reversed MPA's inhibitory effect on p53.
Phosphorylation of p53 normalized to total p53 level in cell lysates. Results represent the mean ± SD compared (n =3). *P < 0.05 relative to vehicle. MPA = mycophenolic acid, OD = optical density, ph-p53 = phosphorylated p53, tot-p53 = total p53.

These results imply that the cytostatic effect of MPA in ECFC is through guanosine depletion via IMPDH inhibition, and the addition of exogenous guanosine rescues MPA-induced inhibition in cell proliferation and senescence.

2.5 Discussion

MPA has potent anti-angiogenic properties and exerts a dose-dependent inhibition of ECFC proliferation *in vitro* by driving cells towards senescence. MPA treatment severely impairs the ability of ECFC to form blood vessels *in vitro* and decreases ECFC cellular migration.

The in vitro results are consistent with previously reported data that MPA/MMF treatment at 1-100 μM concentrations inhibits proliferation and blocks angiogenesis *in vivo* in various types of mature EC [83, 84, 128]. From this work, we conclude that MPA exerts direct effects on proliferative endothelial cell populations that have reparative and regenerative potential. Results of studies evaluating EPC number and function in patients with CIRD, including systemic lupus erythematosus, systemic scleroderma and primary systemic vasculitis, are inconsistent [25-28]. One reason is because there are no EPC-specific cell markers and hence, there is difficulty in accurately defining EPC identity. Many studies purportedly examining EPC number and function were in fact analyzing proangiogenic hematopoietic cells, which are circulating bone marrow-derived cells that plays a role in neovascularization of damaged tissue but do not fully integrate as true endothelial cells into the remodeled endothelium [29-32]. Recent work has highlighted the multi-cellular network of proangiogenic cells and vascular endothelial cells required to effectively repair damaged endothelium [32]. Early work from our group showed that ECFC display all features of the originally defined endothelial progenitor cell, and the infusion of ECFC into pre-clinical animal models of hind limb ischemia, myocardial infarction and retinal ischemia enhanced vascular recovery [19, 33-35]. Due to ECFC's vessel forming potential and the importance of maintaining EC homeostasis, the inhibitory effect of MPA observed in this study highlights the concern for vascular off-target toxicity of this immunosuppressive agents. Further clinical studies are required to

determine first, the intrinsic behavior of ECFC in patients with CIRD and second, whether the drugs exert similar *in vivo* effects on ECFC in rheumatic patients maintained on immunosuppressive therapies. Since these cells are rare in the peripheral circulation [19], analysis will require sampling a high volume of blood from patients which is very challenging in the pediatric population. It is important to note that we started with a concentration range of MPA (1-5 μM) that includes clinically relevant and targeted serum trough concentration of 1-3.5 μg/mL (3.3-11.5 μM).The concentration range was reduced by 10-fold in subsequent experiments due to the potent inhibitory effects of MPA on ECFC *in vitro*. This raises concern that MPA at current therapeutic doses in patients may possess potential vascular toxicity especially with the notable narrow therapeutic index of MPA and large intra- and inter-individual variation in both MPA plasma concentrations and levels of IMPDH activity [36, 37].

We showed that MPA-treated ECFC in vitro display senescent-like features such as lack of proliferation, cell cycle arrest, and increased SA-ß-galactosidase activity. The p53/p21 signaling pathway known to be a critical regulator of cell survival is also activated in a senescence process that subsequently leads to vascular dysfunction [38]. We demonstrated a higher level of expression of the CDK inhibitor $p21^{WAF}$ and p53 activation. In the cardiovascular literature there is accumulating evidence that implicates endothelial cell senescence in the initiation and progression of atherosclerosis. Vascular senescence had been identified in human atherosclerotic lesions and is thought to be an important contributor to the pathogenesis of age-associated conditions like heart failure and diabetes [39-41]. Many adult and childhood-onset systemic rheumatic diseases are complicated by endothelial dysfunction, premature atherosclerosis and increased CV disease that are not fully explained by traditional risk factors and the risks persist even after disease control is clinically attained [2, 5]. Immune related cells undergoing

senescence may be a desired effect to control overactive lymphocytes, or unwanted breakdown in self-tolerance favoring the development of autoimmune disease [42-44]. The possibility that senescent changes in EC may contribute to the pathophysiology and increased CV morbidity of rheumatic disease is plausible and has not been fully examined. Oxidative stress, pro-inflammatory cytokines, enhanced telomere shortening with inadequate repair, and the destructive influence of senescence-associated secretory phenotype are some of the hypothesized mechanistic processes potentially involved [45-47]. It is beyond the scope of this study to investigate the specific regulatory mechanisms and pathways that occur after MPA treatment of ECFC. However, it will be an interesting area to pursue in future research. In our work, MPA caused senescence without induction of apoptosis in ECFC, a finding in contrast to what has been observed in T cells [78, 129].

We added guanosine to assess for IMPDH independent GTP synthesis indirectly. Exogenous guanosine supplementation restored ECFC proliferation and interestingly, it reversed the senescence effect of MPA at a transcriptional and functional level. This finding proves that despite the ability of EC to scavenge free purine and/or purine-containing ribonucleosides and deoxyribonucleosides to maintain critical cellular levels of these essential metabolites [50], the guanine salvage pathway is unable to compensate for the low GTP pool to prevent cell growth arrest in MPA treated cells.

In conclusion, our study demonstrates that MPA has a potent inhibitory and anti-angiogenic effect on EC. Here, we provided a plausible molecular mechanism in the senescence pathway not previously demonstrated. MPA drives ECFC to go into premature senescence and thus is likely to have pathophysiologic significance in the early atherosclerosis seen in CIRD. The direct short- and long-term clinical consequence

of these effects on cardiovascular health and morbidity are unknown. This warrants a better understanding of how other immune suppressive drugs disrupt vascular homeostasis and might contribute to endothelial dysfunction. In the clinic, MMF and MPA are favored by many rheumatologists due to their steroid-sparing properties, reversible side effect profile, ease of administration and renal protective effects [130-132]. However, recognition and understanding of the mechanisms of this endothelial toxicity will be crucial for optimal patient management. This study highlights the need for more translational research to gain insight into mechanistic links among endothelial cell biology, systemic inflammation, and vascular injury and repair in patients with rheumatic disorders. Further knowledge can usher the discovery of better and safer immunosuppressive therapies and drug regimens in treating patients with systemic rheumatic disease that optimize desired effects while minimizing unwanted drug toxicities, the basis for individualized therapy.

CHAPTER III

3.1 SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

3.1.1 EPIDEMIOLOGY OF SLE

SLE is an autoimmune disease of unknown etiology defined by chronic inflammatory processes and the production of a wide range of antibodies against self-antigens. It has an incidence of 2 to 7.6 per 100,000 people per year and a prevalence of 15 to 50 per 100,000 population per year. It primarily affects women and has a female: male ratio of 9:1 [133]. Clinical presentation is highly variable; the disease follows a relapsing and remitting course. SLE is diagnosed based on fulfilling at least 4 out of the 11 criteria proposed by the American College of Rheumatology (ACR) in 1997 (Table III-1) or 4 of the 17 SLICC/EULAR classification criteria, with at least one clinical and one immunologic criterion (Table III-2).

Clinical	**Laboratory**
Malar rash	Hematologic disorder - hemolytic anemia, lymphopenia, leukopenia, thrombocytopenia
Discoid rash	Immunologic disorder - anti-DNA, anti-Sm, anti-phospholipid Ab
Photosensitivity	Antinuclear antibody
Oral ulcers	
Arthritis	
Serositis	
Renal disorder	
Neurological disorder	

Table III-1. The 1997 Updated ACR Classification Criteria for SLE

Clinical Features	Immunologic Features
Acute cutaneous lupus (maculopapular lupus rash, malar rash, photosensitive lupus rash)	Anti-nuclear antibody
Chronic cutaneous lupus (discoid rash, mucosal lupus)	Anti-DNA
Oral or nasal ulcers	Presence of Anti-Sm
Non-scarring alopecia	Anti-phospholipid antibody
Arthritis or synovitis in ≥ 2 joints	Low complements (C3, C4, CH50)
Serositis	Direct Coombs' test
Renal disorder (urine protein or RBC casts)	
Neurological disorder (seizures, psychosis, others)	
Hemolytic anemia	
Leukopenia or lymphopenia (without an identifiable cause)	
Thrombocytopenia (without an identifiable cause)	

Table III-2. The 2012 SLICC/EULAR Classification Criteria for SLE

3.1.2 RENAL INVOLVEMENT IN SLE

Lupus nephritis (LN) is a common manifestation of SLE. The clinical manifestation may be subtle, suspected when proteinuria and/or hematuria is present. A definitive diagnosis of LN is established with a kidney biopsy. LN has a profound negative impact on kidney

and overall survival across age-groups [134]. Immune-complex LN is described pathologically using the 2003 International Society of Nephrology/Renal Pathology Society (ISN/RPS) nomenclature. It classifies LN on the basis of where immune complexes accumulate in glomeruli, the presence or absence of mesangial or endocapillary proliferation, the overall extent of glomerular involvement (focal or diffuse) and glomerular injury (global or segmental), and whether glomerular injury is active (inflammatory) or chronic (sclerotic) (Table III-3) [135]. In general, the proliferative classes are treated aggressively, whereas non-proliferative classes can be managed more conservatively. Aggressive immunosuppressive therapy has improved the prognosis of SLE patients with renal disease considerably, but 5-20% still progress to end stage renal disease (ESRD) within 10 years following the diagnosis of nephritis [136]. Patients with LN have a higher standardized mortality ratio and die earlier than SLE patients without LN [137].

Although immune-complex–mediated glomerulonephritis is the most common cause of kidney disease in SLE, there are other mechanisms that result in renal injury. Renal vascular complication is another striking feature of lupus and this could include uncomplicated vascular immune deposits, non-inflammatory necrotizing vasculopathy, thrombotic microangiopathy, true renal vasculitis, thrombotic thrombocytopenic purpura, antiphospholipid antibody syndrome and renal vein thrombosis [4].

Class I	**Minimal mesangial lupus nephritis** Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence
Class II	**Mesangial proliferative lupus nephritis** Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits May be a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy
Class III	**Focal lupus nephritis**[a] Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations
Class III (A)	Active lesions: focal proliferative lupus nephritis
Class III (A/C)	Active and chronic lesions: focal proliferative and sclerosing lupus nephritis
Class III (C)	Chronic inactive lesions with glomerular scars: focal sclerosing lupus nephritis
Class IV	**Diffuse lupus nephritis**[b] Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental(IV-S) lupus nephritis when ≥50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) lupus nephritis when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation
Class IV-S (A)	Active lesions: diffuse segmental proliferative lupus nephritis
Class IV-G (A)	Active lesions: diffuse global proliferative lupus nephritis
Class IV-S (A/C)	Active and chronic lesions: diffuse segmental proliferative and sclerosing lupus nephritis
	Active and chronic lesions: diffuse global proliferative and sclerosing lupus nephritis
Class IV-S (C)	Chronic inactive lesions with scars: diffuse segmental sclerosing lupus nephritis
Class IV-G (C)	Chronic inactive lesions with scars: diffuse global sclerosing lupus nephritis
Class V	**Membranous lupus nephritis** Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations Class V lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed Class V lupus nephritis show advanced sclerosis
Class VI	**Advanced sclerosis lupus nephritis** ≥90% of glomeruli globally sclerosed without residual activity

[a] Indicate the proportion of glomeruli with active and with sclerotic lesions.
[b] Indicate the proportion of glomeruli with fibrinoid necrosis and/or cellular crescents.
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.

Table III-3. International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis**.** Adapted from Weening *et. al.* (2004) [135].

3.1.3 CARDIOVASCULAR DISEASE (CVD) IN SLE

Mortality in SLE follows a bimodal pattern. Urowitz *et. al.* in 1976 showed that early death is related lupus disease activity and infection, and this is followed by a second later peak mostly due to CVD [138]. This pivotal study made physicians aware that SLE is associated with premature atherosclerosis, coronary artery disease, myocardial infarction and stroke. Several papers from other research groups later showed varying risk estimates but a consistent trend of significantly increased risk of CVD in patients with SLE compared to healthy controls [139-141]. It is known that CVD risk factors of obesity, hypertension, and dyslipidemia is more prevalent in SLE patients. However, traditional CV risk factors do not completely account for the heightened CV risk [142], therefore it is likely that lupus-related risk factors like chronic inflammation, cytokines and organ damage, are an important contributors and may play a more vital role in CV risk development in patients with SLE. Despite a significant reduction in mortality among lupus patients over the last 5 decades with current 5-year survival of more than 85%, it is interesting to note that lupus-related mortality has decreased in all categories except CVD [140, 141].

3.1.4 CHILDHOOD-ONSET SLE (cSLE)

Childhood-onset SLE, with disease onset prior to 16 years of age, represents 15-20% of SLE patients [143]. Children and adolescents generally have more severe illness at initial presentation, more aggressive disease course, higher rates of organ involvement, accrue greater disease damage over time, and have increased need for long-term immunosuppressive medication [144, 145].

Renal disease is more severe and more common in cSLE. It occurs in 50 to 75% of all cSLE patients, mostly within the first 2 years after diagnosis, with 18 to 50% of them

progressing to ESRD. Several cohort studies report a higher percentage of pediatric patients who have biopsy-proven renal disease, with a trend toward significantly more proliferative nephritis than adults [145-147].

Given their lifelong exposure to atherogenic risk factors, children and adolescents with SLE are at particularly high risk for developing premature atherosclerosis. Studies have shown that compared to healthy controls, there is evidence of structural and functional endothelial dysfunction and damage in cSLE [148-151].

3.2 ENDOTHELIAL CELL (EC) DAMAGE LEADING TO TISSUE INJURY

Steroids and immunosuppression are the cornerstone in management of lupus. While advances in immunosuppressive treatment have decreased organ damage and life-threatening events in SLE, there is still no cure for SLE and not all patients respond to immune suppression. This reflects incomplete understanding of disease pathogenesis and the need find new treatment target.

In the kidneys, EC interact with mesangial cells, podocytes, renal tubular cells and pericytes, all of which are important for cell development, homeostasis, function and survival through release of cytokines, chemokines and soluble factors. When any one of the renal components is damaged, it is shown that ECs are also affected [152-154]. Disturbance of angiogenesis reported in LN is associated with endothelial dysfunction including a decrease in the ratio of pro-angiogenic Ang1/anti-angiogenic Ang2, downregulation of the angiogenic factor FGF-2, an increase in the VEGF inhibitor ADAMTS-1, and alterations in endothelial nitric oxide synthase [155-157].

EC are mainly affected in SLE-associated CVD. High type I interferon (IFN) production and the pro-inflammatory environment seen in SLE patients affect the endothelial cell population, and appears to play a critical role in tilting the balance towards endothelial damage and impaired vascular repair [120, 158]. Other known factors toxic to endothelial cells including activated inflammatory cells, immune complexes, expression of costimulatory molecules, generation of neutrophil extracellular traps (NETs), lupus-related autoantibodies, oxidative stress, and dyslipidemia, likely tip the balance towards endothelial dysfunction and propensity to develop premature cardiovascular disease in lupus patients.

3.3 PRISTANE INJECTION AS A MURINE MODEL OF SLE DISEASE

Pristane (2,6,10,14 tetramethylpentadecane or TMPD) is a hydrocarbon found in many plants, marine organisms, crude oil and is a common constituent in mineral oil used in food, cosmetic and pharmaceutical products. When pristane is given parenterally to non-autoimmune prone mice, it results in the generation of autoantibodies and development of clinical manifestations associated with SLE [159]. It had been repeatedly shown that ANA, anti-ssDNA, anti-dsDNA, anti-Su, anti-Sm, anti-RNP, anti-histone and anti-ribosomal P, auto-antibodies common in human SLE, develop within 1-6 months after intraperitoneal injection of pristane [159, 160]. Treated mice develop features of lupus syndrome such as arthritis, serositis, glomerular hypercellularity, glomerular immune complex deposition and proteinuria. A unique feature of this murine model is that it is the only one reported to have the interferon (IFN) signature characteristic of human SLE. IN humans, SLE is associated with excess and dysregulated production of type I IFN that is reflected in overexpression of IFN-I-stimulated genes. This pattern is known as the interferon signature and it is clinically important since a correlation has been found with disease activity, renal involvement, and the presence of autoantibodies [161]. Peripheral

blood mononuclear cells from mice injected with pristane overexpress interferon signature genes; moreover, signaling via the IFN alpha receptor is central to the pathogenesis of lupus in this animal model [162, 163].

3.4 VASCULAR ENDOTHELIAL STEM CELL (VESC) AND VESSEL REGENERATION

Although it is clear that a subset of EC has clonal proliferative potential and contributes to postnatal vasculogenesis, we do not know where these cells arise from. It is also unclear how the EC pool is replaced after injury and maintained during homeostasis, i.e., health. More recently, there is evidence to suggest that endothelial stem cells give rise to mature EC and reside in the vascular wall. Stem cells have an extensive capacity to proliferate, differentiate into mature tissue-specific cells, and self-renew, enabling them to repopulate recipients after cellular transplantation [164, 165]. Much effort has been devoted to isolating and characterizing adult stem cells that give rise to the vascular endothelium including: side population method which is a flow cytometry technique used to obtain stem cells based on the dye efflux properties of the ATP-binding cassette (ABC) transporters [166-168], and use of cell surface protein markers like c-Kit, protein C receptor and CD157 [169-171]. However, the side population technique is based on function rather than a cell surface markers, making it difficult to be used to prospectively identity these cells in vivo. Existing markers are inadequate because they are either non-specific, are not detected in other tissues, or not shown to be present in human EC. Despite progress in discovering new stem cell markers, better methods are needed for identifying postnatal vascular endothelial stem cells (VESC).

3.5 ABCG2 TRANSGENIC MOUSE MODEL

The ABC family drug transporter Abcg2 has been associated with the side population phenotype and stem cell activity in various organs [172-174]. The Yoder lab explored the

Abcg2 as potential VESC marker in a murine system using the Abcg2 transgenic mouse model.

Genetic recombination is the most widely used labeling system in mouse models. This technology utilizes tissue or cell-specific promoters that control Cre recombinase enzyme expression which when activated, promote a reporter transgene by the excision of a loxP-flanked transcriptional stop cassette [175]. Using an inducible system like this, Cre is activated only in the presence of an anti-estrogen or progesterone receptor agent. This allows tracking the fate of cells over time [176]. Fatima et al. generated a lineage tracing mouse model based on an allele that co-expresses both Abcg2 and a CreERT2 (Estrogen Receptor Type 2) expression cassette (*Abcg2CreERT2*) to track Abcg2-expressing cells and their progeny in many organs [177]. We bred mice transgenic for tamoxifen inducible *Abcg2CreERT2* with *ROSATdTomato* mice to generate ABCG2TT mice that identify *Abcg2*-expressing EC in the murine system. Unpublished data from Yoder lab reveals that Abcg2-expressing EC play a role in new blood vessel formation in the heart, lungs, muscle, retina and skin. More importantly, these endothelial cells fulfill all the criteria of VESC including clonal proliferative potential, ability to self-renew and contribution to multiple blood vessel compartments (artery, vein, capillary) upon transplantation via fate mapping analysis. This system will allow the investigation of the characteristics of resident VESC in different tissues following various types of injury. Particularly, this model will allow us to investigate how chronic rheumatic inflammatory disease (CIRD) like SLE and relevant immunosuppressive treatments impact the nature and functions of EC with reparative and regenerative potential.

I hypothesize that kidney RVESC display differential reparative responses depending on the nature of the injury.

3.6 MATERIALS AND METHODS

3.6.1 MICE

C57BL/6 and R26R-TdTomato mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA), housed and bred in a conventional facility at Indiana University. Colony of ABCG2CreERT mice used by Fatima *et. al*. were recovered from sperm provided by Dr. Sorenttino at Indiana University School of Medicine Laboratory Animal Resource Center.

3.6.2 TAMOXIFEN INJECTION

Tamoxifen (Sigma-Aldrich) was suspended in sunflower seed oil (Sigma-Aldrich) at 37°C to make a 4mg/ml solution and was stored at -20°C until use. To induce Cre expression in ABCG2CreERT mice, 50mg/kg tamoxifen was injected into the animals intra-peritoneally (i.p.) at appropriate time points.

3.6.3 TISSUE CELL ISOLATION

Single cell suspensions from heart and kidney were prepared by mincing the tissue, digesting with 0.25% collagenase I (Stem Cell Technologies) at 37°C for 30-40 minutes, and disruption of cell clumps over 70 μm cell strainer (Falcon).

3.6.4 FLOW CYTOMETRY

The following conjugated antibodies were used: anti-CD31-PECy7, anti-CD45-APC, and Ter119-FITC were purchased from eBioscience. 1:1000 propidium iodide (PI, Sigma-Aldrich) was added to sorting buffer before analysis to label dead cells. Cells were analyzed for $PI^{-}Ter119^{-}CD45^{-}CD31^{+}TdTomato^{+}$ (*Abcg2*-expressing EC) using a FACSAria flow cytometer (BD Biosciences).

3.6.5 COLONY FORMING ACTIVITY

CD31+ CD45- cells were sorted using Magnetic Activated Cell Sorting method. Isolated murine EC were re-suspended in EC culture medium (alpha-MEM with 10% FBS [Hyclone], 5 × 10−5 M β-mercaptoethanol [Sigma-Aldrich] and 0.5% penicillin/streptomycin [Gibco]), plated on a bed of OP9 stromal cells in limiting dilutions, and then incubated at 37°C, 5% CO_2. Non-adherent cells were removed the next day and media changed every 3 days for 14 days. Plates were fixed with 4% paraformaldehyde at room temperature, washed with PBS, blocked with 2% skim milk (Sigma-Aldrich) in 0.1% triton (Sigma-Aldrich) PBS solution (PBSMT solution) for 30 minutes at RT and then stained with 1:100 rat anti mouse CD31 (BD Pharmingen) 4°C overnight, stained with 1:200 alkaline phosphatase conjugated donkey anti rat IgG secondary antibody (Jackson ImmunoResearch) in PBSMT at RT for 2 hours, and lastly with DAPI staining. Colonies were counted and images captured using Leica™ DM IL microscope with a SPOT RT3 camera.

3.7 PRELIMINARY RESULTS

In neonates, 8.3±1.7% of total EC expressed TdTomato one day after tamoxifen injection. After 7 days, this population contributed to 36.0±11.1% of all kidney EC. In adult mice, 2.9±1.3% of total EC was labeled one day after a single tamoxifen injection. This population contributed to 15.4±2.9% of total kidney EC after 84 days (Figure III-1).

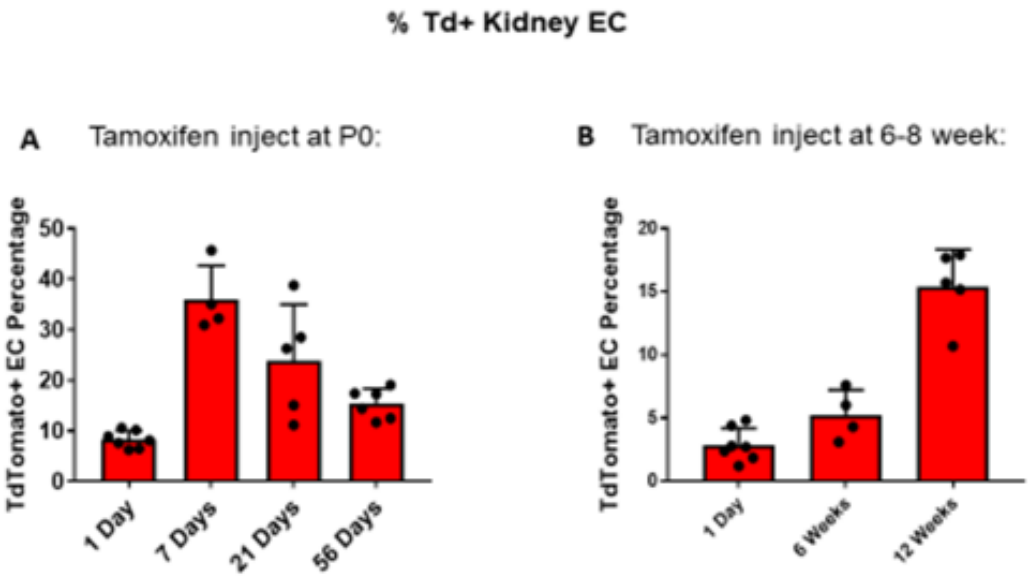


Figure III-1. Kidney VESC lineage tracing.

Quantification of lineage tracing experiment with flow cytometry shows that Abcg2-expressing VESC contribute to kidney vessel growth in neonates (A) and tissue maintenance in adults (B). Data represent mean ± S.D.

In neonatal heart, 18.96±5.5% of total EC expressed TdTomato one day after tamoxifen injection. After 7 days, this population contributed to 66.0±3.1% of all heart EC. In adult mice, 9.8±5.3% of total EC was labeled one day after a single tamoxifen injection. This population contributed to 8.3±2.9% of total heart EC after 84 days (Figure III-2).

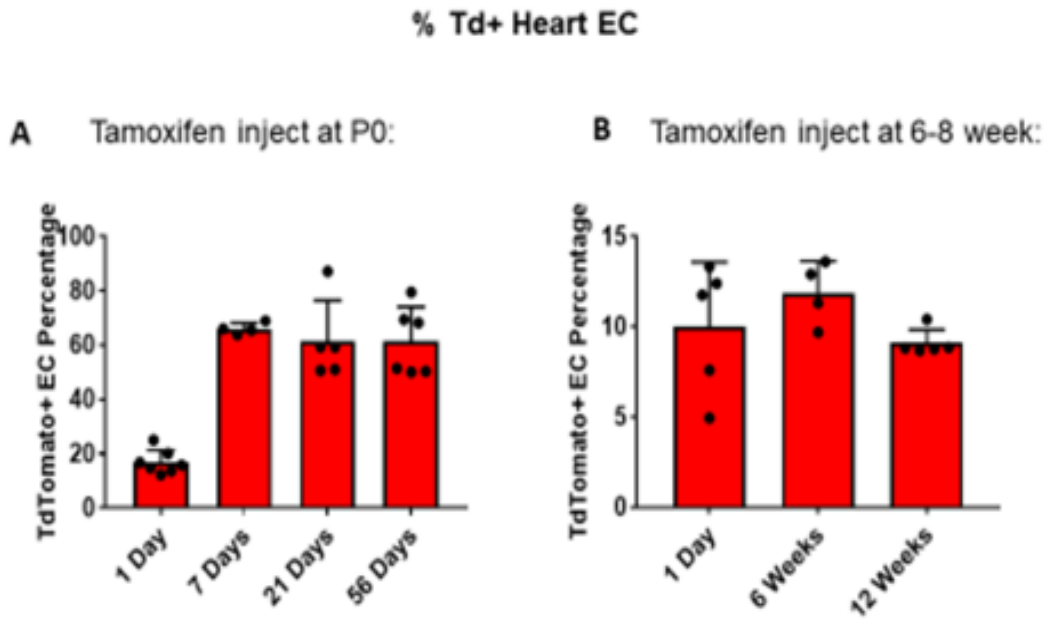


Figure III-2. Heart VESC lineage tracing.

Quantification of lineage tracing experiment with flow cytometry shows that Abcg2-expressing VESC contribute to heart vessel growth in neonates (A) but do not change significantly with time when measured in adults (B). Data represent mean ± S.D.

The frequency of colony formation of TdTomato labeled EC is significantly higher than non-labeled EC (Figure III-3 and III-4).

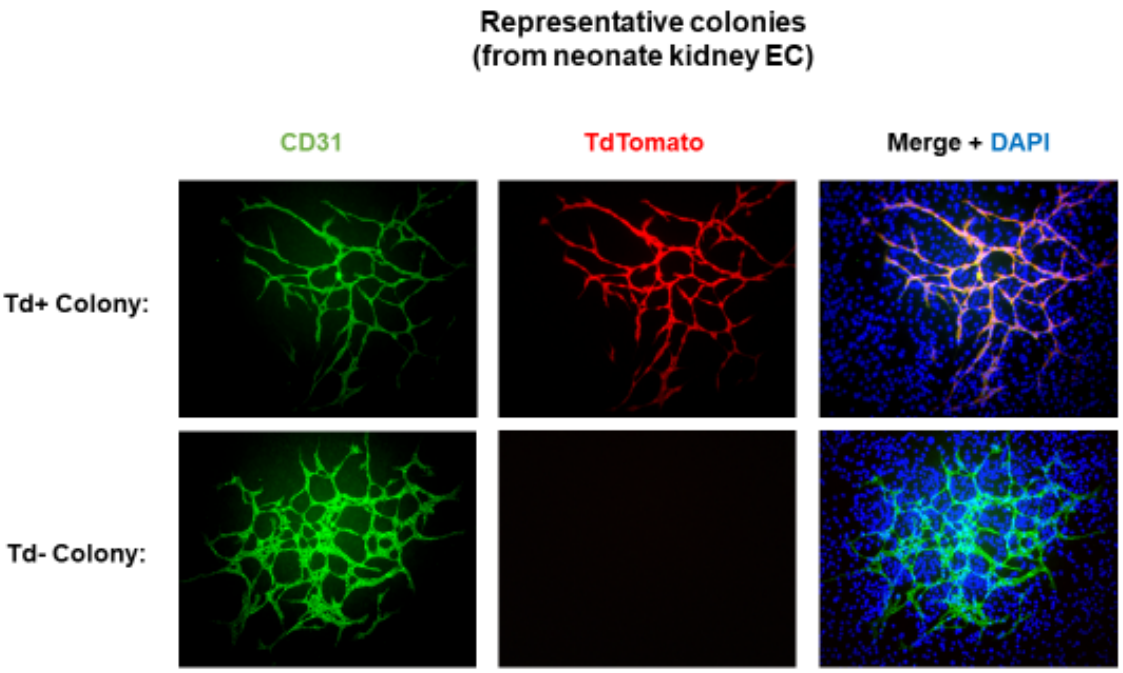


Figure III-3. Neonatal kidney EC colonies

Representative pictures of EC colonies derived from TdTomato+ EC (top panels) and TdTomato- EC (bottom panels) from kidney of P7 ABCG2TT mice 24 hours after single tamoxifen injection.

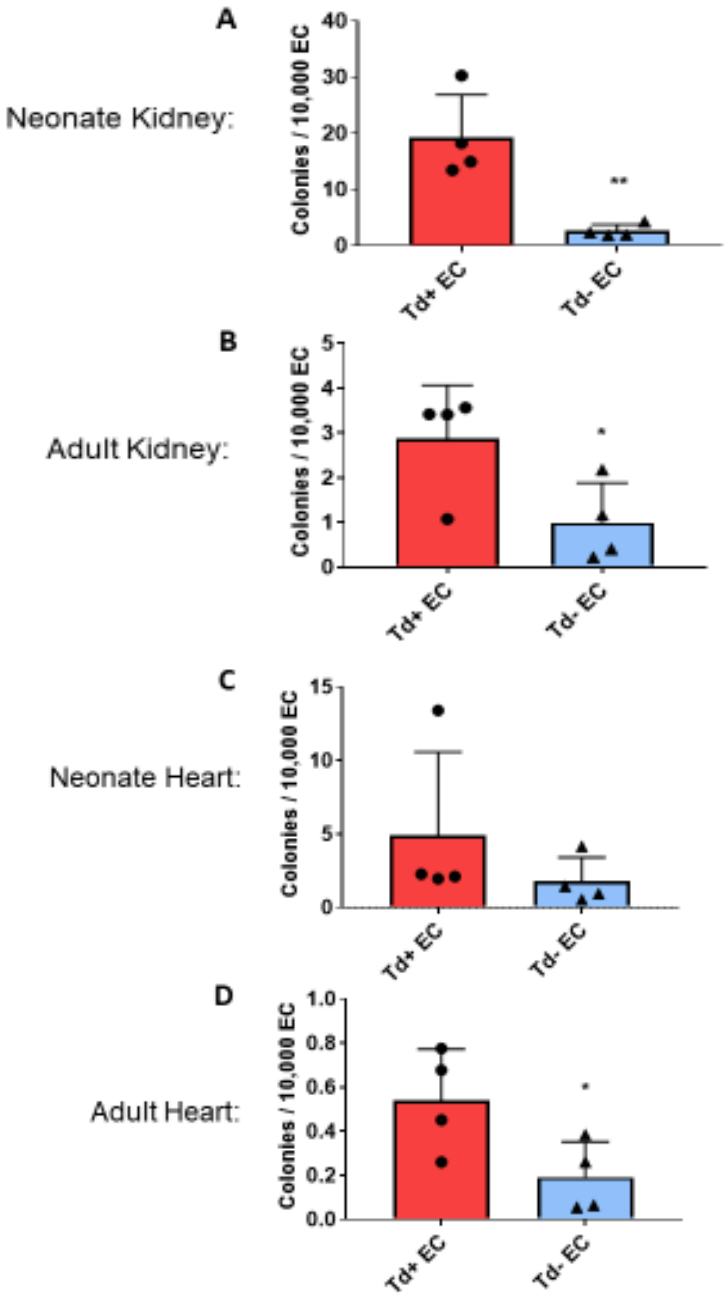


Figure III-4. Colony Forming Assay of Kidney and Heart EC.

Frequency of TdTomato+ and TdTomato- EC colonies derived from neonate (A) and adult (B) kidneys, compared with neonate (C) and adult (D) heart 24 hours after a single tamoxifen injection. Data represent mean ± S.D. *p<0.05

3.8 HYPOTHESIS AND AIMS

I hypothesize that pristane-injected mice display altered endothelial features in tissues that show features of SLE disease in the pristane-induced murine SLE model.

The aims of future study are to:

Aim 1: Characterize the changes in EC features observed in pristane-induced lupus

- To characterize the extent of endothelial cell injury in heart, lungs and kidneys
    - Frequency of CD45-CD31+ cells by FACS analysis at different time points
    - Microvessel density and EC frequency by histology and immunostaining
    - EC activation, inflammation and damage by gene and protein expression (cytokines, chemokines, adhesion molecules)
- To assess VESC proliferative potential and activity in heart, lungs and kidneys
    - Frequency of CD45-CD31+ TdTomato+ cells
    - Colony forming activity of CD45-CD31+ TdTomato+ cells
- To identify correlation between lupus disease activity, EC and VESC behavior
    - Measure serum antibody and interferon levels
    - Organ specific markers of lupus disease (serum creatinine, proteinuria, histology, markers of IFN-α activity like IP-10 and/or MCP1)

Aim 2: Investigate the in vivo effects of MMF in mice

- To assess the tissue resident VESC survival in the heart, lungs and kidneys of ABCG2TT mice after MMF treatment
    - Frequency and colony forming activity of CD45-CD31+ TdTomato+ cells using lineage tracing technique

- Cell cycle, apoptosis, senescence by FACS, histology, gene and protein expression

- Determine the efficacy of MPA/MMF in treating pulmonary hemorrhage and nephritis in pristane-induced lupus
  - Serum auto-antibodies and interferon concentrations and histology
  - Tissue resident VESC proliferation and activity

3.9 EXPERIMENTAL DESIGN

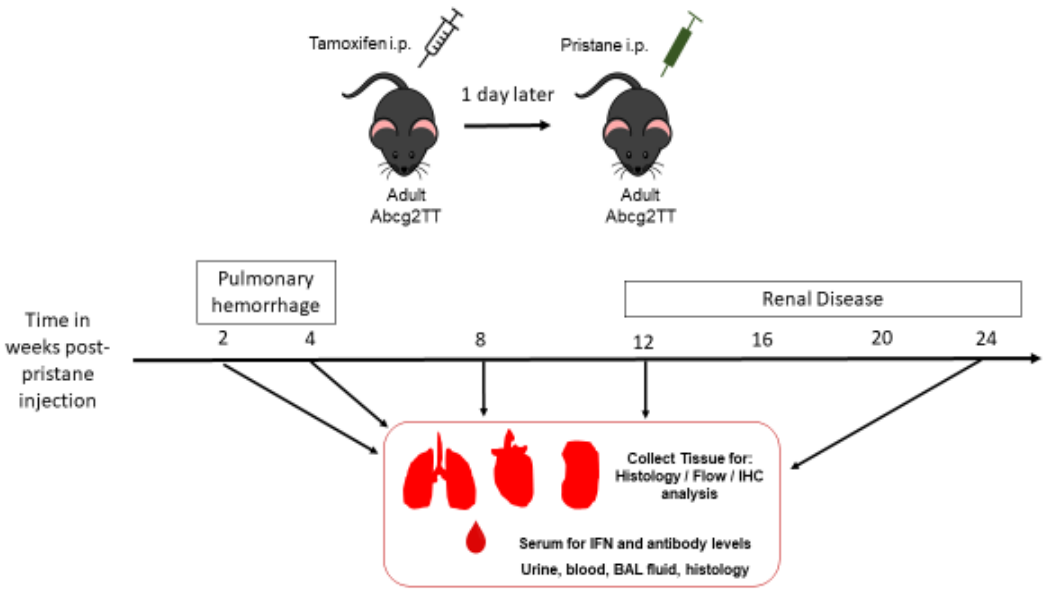


Figure III-5. Timeline for Aim 1

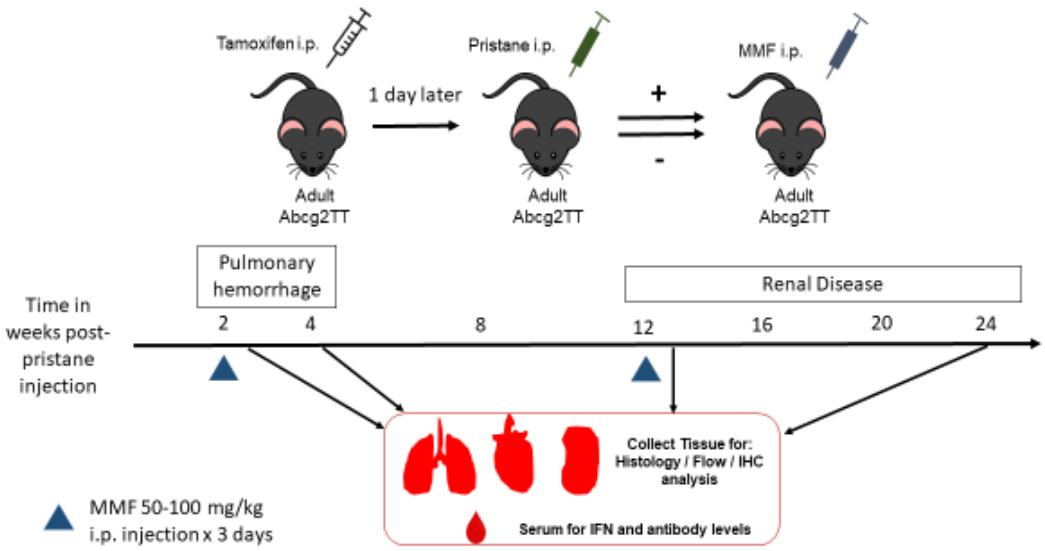


Figure III-6. Timeline for Aim 2

3.10 SIGNIFICANCE

This study will further our understanding of the vascular involvement in SLE which is necessary for the development of specific cellular and targeted treatment strategies. Knowing how to identify these cells should make it possible to manipulate endothelial cell proliferation, which in turn should aid in the development of therapies to promote vascular repair. Knowledge obtained from this study can be extended to other rheumatic disease that also have prominent vasculopathy or vasculitis. We can also use the same system to look at how other immunosuppressive agents or combined therapies influence tissue-specific blood vessel endothelial health, repair and regeneration in relation to existing autoimmune/rheumatic disease.

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CURRICULUM VITAE

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Lupus Foundation of America	Washington DC, USA	2013-2016
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ABSTRACTS

Go E, Ballinger S, Klausmeier T. “A Case of A20 Haploinsufficiency (HA20)”, Riley Hospital for Children 13th Pediatric Scholars Day, Indianapolis, IN, May 2016

Go E, Tarvin S. “A case of arthritis and parenchymal lung disease in a pediatric patient”, Academic Pediatric Association, Indianapolis, IN, March 2017

Go E, O’Neil K, Yoder M. “Mycophenolic Acid inhibits endothelial cell migration, proliferation and function in vitro”, 18th International Vasculitis and ANCA Workshop, Tokyo, Japan, March 2017

†Banschbach K, **Go E**, Tarvin S. “Clinical Outcomes of Juvenile Dermatomyositis Patients Treated with TNF-Inhibitors: A Retrospective Chart Review”, American College of Rheumatology Scientific Meeting, San Diego, CA, November 2017

Go E, Lin Y, Yoder MC, Basile DB. “Abcg2-expressing endothelial cells contribute to kidney postnatal vascular growth and maintenance”, American Society of Nephrology Meeting, San Diego, CA, October 2018

PUBLICATIONS

Go, Ellen J.L.; O’Neil, Kathleen M. The Catastrophic Antiphospholipid Syndrome in Children. Current Opinion in Rheumatology: September 2017 - Volume 29 - Issue 5 - p 516–522. doi:10.1097/BOR.0000000000000426

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